Regulation of autophagy and cell death in breast carcinoma cells

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A Dissertation

entitled

Regulation of Autophagy and Cell Death in Breast Carcinoma Cells

by

Kristen L. Koterba

Submitted to the Graduate Faculty as partial fulfillment of the requirements for the

Doctor of Philosophy Degree in Biomedical Sciences

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Mammalian intracellular protein homeostasis is maintained by a process called macroautophagy, or autophagy, and is a major protein degradation mechanism induced by cellular stress such as nutrient starvation. The discovery of key autophagy genes as well as signaling pathways involved in the regulation of autophagy has elucidated the essential protein machinery involved in autophagosome formation, fusion, and degradation. However, the specific mechanism as to how these genes and kinase pathways regulate autophagy is largely unknown. In addition to normal cellular physiology, autophagy has been implicated in the pathophysiology of cancer. This is evidenced by an increase in the frequency of spontaneous malignancies by heterozygous disruption of the autophagy gene Beclin-1; suggesting that Beclin-1 functions as a tumor suppressor gene. We show that Beclin-1 co-immunoprecipitates with the Class III PI3K hVps34 and its myristoylated protein adapter p150, and that this interaction requires amino acids 80-107 of Beclin-1. We also demonstrate that upon autophagic stimulation, the Beclin-1-hVps34-p150 complex is recruited to the membrane fraction in MCF-7 cells.
Rottlerin is a cytotoxic compound reported to increase the presence of autophagic vacuoles in the cytoplasm of pancreatic cells, membrane association of GFP-tagged LC3 to autophagosomes, and a marked induction of LC3-II protein, all important hallmarks of autophagy. Additionally, inhibition of Beclin-1 expression by RNAi was shown to inhibit Rottlerin-induced autophagy. Rottlerin was initially reported to specifically inhibit Protein Kinase C delta (PKCδ), and has also been shown to uncouple mitochondrial oxidative phosphorylation. The second portion of the dissertation aimed to explore the hypothesis that the cytotoxic effects of Rottlerin are related to its regulation of autophagy. Our results demonstrate that Rottlerin causes an accumulation of the autophagosomal marker LC3-II in MCF-7 and ZR75 breast cancer cells. This increase in accumulation of LC3-II was caused mainly by an impairment of the autophagy pathway at the lysosomal degradation step. However, a modest increase in autophagosome biogenesis contributes to the accumulation of LC3-II. Our studies concluded that exposure of breast cancer cells to Rottlerin induces caspase-independent cell death by reducing mitochondrial membrane potential (MMP), stimulating ER stress, and inhibiting autophagosome fusion with lysosomes, thereby rendering the autophagy survival pathway ineffective.
I dedicate this dissertation to my wonderful family: those that are still here, and those that I have lost. I am forever grateful for their love and support in all that I do.
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Chapter 1

Regulation of Autophagy by the Beclin-1/hVps34/p150 PI3-Kinase Complex

1.1 Introduction

Mammalian cellular protein homeostasis is maintained by a process called macroautophagy, or autophagy, which is a major intracellular protein degradation mechanism induced by cellular stress such as amino acid or growth factor starvation. Autophagy is a normal physiological mechanism for the degradation of long-lived proteins and organelles. The degradation and turnover of these proteins yields amino acids that can be recycled for cell survival. Defects in autophagy can play a significant role in human pathologies including cancer, neurodegeneration, and infectious diseases (Heath and Xavier, 2009). The discovery of key autophagy genes as well as signaling pathways involved in the regulation of autophagy has elucidated the essential protein machinery involved in autophagosome formation, fusion, and degradation (Huang and Klionsky, 2002; Klionsky and Emr, 2000; Reggiori and Klionsky, 2002). However, the specific mechanisms as to how these genes and kinase pathways regulate autophagy are largely unknown. In addition to normal cellular physiology, autophagy has been
implicated in the pathophysiology of cancer, but there are arguments as to whether the role of autophagy in cancer is a pro-death or pro-survival mechanism. Experimental evidence supporting autophagy as a pro-death mechanism includes an increase in the frequency of spontaneous malignancies by heterozygous disruption of the autophagy gene Beclin-1 (Aita et al., 1999). This suggests that Beclin-1 functions as a tumor suppressor gene. Additionally, Beclin-1 expression is markedly reduced in human cancer cells compared to normal cells (Chen et al., 2009; Won et al., 2009). These data together indicate an essential role of autophagy genes, including Beclin-1, possibly through protein-protein interactions and/or alterations in autophagy-regulatory signaling pathways in human cancers.

Phosphatidylinositol 3-phosphate (PI3P) is a product of hVps34, a Class III PI3-kinase, and is required for the early stages of autophagic vesicle formation (Gillooly et al., 2000; Vieira et al., 2001). Proteins containing FYVE or PX domains can interact with PI3P. Thus, generation of the phospholipids by hVps34 may lead to autophagy by recruitment of proteins necessary for formation of the autophagosome. hVps34 binds to a myristoylated serine/threonine protein kinase p150, which serves as an adaptor protein at the autophagosomal membrane and enhances its kinase activity (Panaretou et al., 1997). Beclin-1 is a known interacting partner of hVps34 (Furuya et al., 2005). PI3-kinase inhibitors such as 3-methyladenine are able to prevent autophagy induction by Beclin-1, suggesting that Beclin-1 associated hVps34 kinase activity is an essential step in the pathway leading to autophagic cell death. This section of the dissertation tests the central hypothesis that Beclin-1 regulates the interaction and targeting of hVps34 PI3-kinase and
its myristoylated adapter protein p150 to the autophagosomal membrane in breast cancer cells.

To determine the precise role of Beclin-1/hVps34 in autophagy, we first set out to identify protein-protein interactions to gain insight into how these interactions modulate autophagy. Although the interaction between Beclin-1, hVps34, and p150 has been identified in yeast, this interaction had yet to be confirmed in mammalian cells at the time that I began these studies. In a previous study, cytosolic Beclin-1 and hVps34 were found to co-elute in a broad peak with a molecular mass of 500-600kD when subjected to gel filtration chromatography (Zeng, X. and Maltese, W.A., unpublished data).

Consistent with this observation in MCF-7 cells, the 60kD Beclin-1 also co-elutes with the 100kD hVps34 in a much larger complex in U251 glioblastoma cells, indicating that at least in the cytosol this large complex may exist universally and consist of other proteins in addition to Beclin-1 and hVps34. Additionally, shRNA-mediated suppression of Beclin-1 expression in U251 cells caused hVps34 to shift to a molecular complex of 200-300kD, which suggests that suppression of Beclin-1 expression disrupts the large protein complex and possibly creates a pool of hVps34 for use in other molecular mechanisms besides autophagy. To identify other proteins within the Beclin-1/hVps34 complex, we developed a MCF-7 human breast cancer cell model where a tetracycline repressible promoter controls expression of FLAG-tagged Beclin-1. Lysates from MCF-7 FLAG-Beclin-1 cells were immunoprecipitated with anti-Flag M2 affinity gel, and subjected to SDS-PAGE to identify interacting proteins of interest by mass spectrometry. The results of the mass spectrometry identified p150 as an interacting protein that co-
immunoprecipitated with FLAG-Beclin-1 in addition to hVps34. These results were confirmed by immunoblotting with hVps34 and p150 antibodies. Additionally, several other proteins were identified in the complex including Hsp70, PP2A inhibitor, and actin.

At the time these experiments were conducted, we were the first to determine that p150 is part of the mammalian complex of Beclin-1 and hVps34. Subsequent publications by other groups confirmed this interaction and revealed the other components of the autophagosome complex. These components include either the mammalian homolog of the UV radiation resistance-associated gene protein UVRAG or the autophagy protein Barkor/Atg14L, and Beclin-1, hVps34, and p150 (Liang et al., 2007; Matsunaga et al., 2009; Sun et al., 2008; Takahashi et al., 2007). The functional role of Beclin-1 was also revealed as a component of the type-III PI-3’-kinase complex, and that this complex is a specific positive mediator of autophagy and not endosomal trafficking (Itakura et al., 2008; Zeng et al., 2006). In mammalian cells, although many other Beclin-1-binding proteins have been reported, the regulation of Beclin-1 is not well understood. Evidence of multiple Beclin-1-hVps34 complexes or multiple functions associated with the Beclin-1-hVps34 in mammals will help in identifying the physiological role of hVps34 and p150 in autophagy.
1.2 Literature

IDENTIFICATION AND CHARACTERIZATION OF BECLIN-1

Liang et al. were the first to identify Beclin-1 by interaction with Bcl-2 in a yeast two-hybrid screen (Liang et al., 1998). The exon-intron structure of the beclin 1 (BECN1) gene revealed 12 exons, extending over 12kb of the human genome. Translated Beclin-1 encodes a 450 amino acid, 60kD coiled-coil protein, that maps to a region approximately 150kb centromeric to BRCA1 on chromosome 17q21 that is commonly deleted/mutated in breast, ovarian, and prostate cancer. The complete cDNA sequence encodes a 2098-bp transcript with a 120-bp 5’-UTR, 1353-bp coding region, and 625-bp 3’-UTR (Aita et al., 1999).

The GenBank sequence entered was originally described as a 17q21 gene with unknown function before Liang et al. cloned and characterized it as the yeast ortholog to the autophagy gene *Atg6* (Friedman et al., 1995). The mouse and human Beclin-1 share 98% identity at the protein level and contain a Bcl-2 binding domain (residues 88-150, Bcl-2BD), Bcl-2 homology domain 3 (residues 108-127, BH3), and the evolutionarily conserved domain required for association to intracellular membranes (residues 244-337, ECD).
Beclin-1 is ubiquitously expressed at the mRNA and protein level in most tissues including spleen, thymus, kidney, heart, lung, and liver and present at highest levels in human skeletal muscle. Additional alternatively spliced 1.7- and 1.4-kb transcripts have also been observed, although the functions of these splice variants are still unknown (Liang et al., 1998). PROSITE analysis of human Beclin-1 identified several potential glycosylation, phosphorylation, and myristoylation sites but no other functional sequence motifs. It has been observed that DAP-kinase can phosphorylate Beclin-1 at T119, which functions to dissociate binding of Beclin-1 from Bcl-XL thereby promoting autophagy (Zalckvar et al., 2009). The yeast Atg6/Vps30 shares 24.4% amino-acid identity (and 39.1% conservation) with human Beclin-1 (Liang et al., 1999). Vps30 encodes a hydrophilic protein containing 556 amino acids and has a predicted molecular mass of 65kD. Similar to human Beclin-1, the central portion of Vps30 (residues 186 to 322) is predicted to form coiled-coil structures (Lupas et al., 1991). The subcellular localization of Vps30 is partly associated with membrane fractions, while most (~80%) was fractionated in the cytosolic pool (Kihara et al., 2001).

THE ROLE OF BECLIN-1 AS A TUMOR SUPPRESSOR

Beclin 1/Atg6/Vps30 plays an essential role in eukaryotic macroautophagy from yeast to mammalian cells suggesting that autophagy is a highly conserved pathway through evolution. Bcl-2 is an important gene involved in apoptotic regulation by suppressing cell death initiation, making Beclin-1 interaction with Bcl-2 an attractive hypothesis to
explain Beclin-1 tumor suppressor activity. Our group investigated the possibility of a Beclin-1/Bcl-2 interaction, but has found that while interactions between overexpressed Beclin-1 and Bcl-2 could readily be observed, coimmunoprecipitation of the endogenous protein was difficult to detect (Zeng et al., 2006). However, studies that are more recent have demonstrated that interactions do occur between Beclin-1 and Bcl-2, and that such interactions may be important for reciprocal regulation of autophagy and apoptosis (Ku et al., 2008; Levine et al., 2008; Pattingre et al., 2005). Beclin-1 maps to the tumor susceptibility locus on chromosome 17q21 which is monoallelically deleted in 40% to 75% of human sporadic breast, ovarian, and prostate cancer (Aita et al., 1999). A more detailed study by Liang et al. revealed Beclin-1 functions as a tumor suppressor gene by reducing proliferation and clonogenicity in Beclin-1 over-expressing cells as well as reducing tumorigenesis in athymic nude mice (Liang et al., 1999). Additionally, later studies have demonstrated that the heterozygous disruption of Beclin-1 in mice increases the level of spontaneous malignancies and premalignant lesions, increases cell proliferation, and reduces autophagy (Qu et al., 2007).

REGULATION OF MAMMALIAN AUTOPHAGY

Macroautophagy, or autophagy, is a type of membrane trafficking mechanism that delivers cytoplasmic cargo to the lysosome for bulk degradation and recycling under conditions of cell stress. In response to nutrient starvation in eukaryotic cells, a double membrane structure encloses a portion of the cytosol and/or organelles. This structure is termed an autophagosome, and is formed either de novo, or possibly from a dynamic interaction with the endoplasmic reticulum (Abeliovich et al., 2000; Axe et al., 2008;
Hayashi-Nishino et al., 2009; Itoh et al., 2008; Noda et al., 2002). When the autophagosome fully matures, the outer membrane of the autophagosome fuses with the lysosome. The inner membrane of the autophagosome then releases its contents into the lysosomal vacuole where hydrolases disintegrate the membranes of autophagic bodies and its cytosolic contents for reuse (Figure 1-2) (Huang and Klionsky, 2002). Formation of autophagosomes has been extensively studied in both yeast and mammalian cells,
beginning with the earliest screens by yeast two-hybrid analysis to identify genes involved in autophagy. The discovery of key autophagy genes (ATG) in yeast that are involved in the formation of the autophagosomes has led to the classification of these genes into four functional groups in mammalian cells that are involved in induction of autophagy, generation, maturation, and recycling of autophagosomes (Klionsky and Emr, 2000). The first group is an autophagy-regulatory complex consisting of a protein serine/threonine kinase that responds to nutrient availability. This functional group involves the nutrient sensor mammalian target of rapamycin (mTOR) kinase that inhibits autophagy during nutrient abundance. Autophagy proteins that respond through mTOR signaling include Atg1 and Atg13. When nutrients become scarce or cells are treated with rapamycin, mTOR is inactivated, leading to dephosphorylation of Atg1 and induction of autophagy. The second group of genes is a lipid kinase group that controls vesicle nucleation and consists of a myristoylated serine/threonine kinase p150 and the class III phosphatidylinositol-3 kinase Vps34. Autophagosomes are not generated in cells treated with PI3K inhibitors such as wortmannin and 3-MA, indicating the importance of PI3-Kinase activity (Mizushima et al., 2001). Vps34 mediates most of the trafficking events studied through its catalytic product phosphatidylinositol-3-phosphate (PI3P). PI3P binds to proteins that contain FYVE or PX domains, and is required for the formation of the autophagosomal structure (Lindmo and Stenmark, 2006). While this kinase group in S.cerevisiae is present in two complexes, one that controls autophagy together with Atg6/Beclin-1 at the pre-autophagosomal structure, and the other without Beclin-1 that functions primarily in endosome trafficking, in mammalian cells there are three complexes that regulate autophagy (Itakura and Mizushima, 2009; Kihara et al.,
In the Class III PI3K complex, Beclin-1 or possibly another protein in this complex via an autophagic stimulus, is able to divert a subpopulation of hVps34 away from endosomal membrane trafficking to devote to autophagosome formation. Evidence of this is that when cross-linked, all of Beclin-1 is bound to hVps34 whereas approximately 50% of hVps34 is free from Beclin-1 (Kihara et al., 2001), suggesting that under certain conditions, complexes involving different regulatory proteins in the Class III PI3K cascade may modulate autophagic activity. In mammalian cells, Beclin-1 (Atg6/Vps30) coimmunoprecipitates with hVps34 and cycles between the cytosol and membrane (Kihara et al., 2001). hVps34 is a cytosolic protein that becomes associated with endosomal membranes when bound to its myristoylated p150 adaptor protein. It is not known whether p150 anchors hVps34 to autophagic membranes via its myristate group. It is possible that the interaction between Beclin-1 and hVps34 is mediated by the adaptor protein p150. More recently, molecular analyses in mammalian cells showed that amino acids 244–337 of Beclin-1 are required for autophagy and its binding to hVps34 but are not essential for vacuolar protein sorting and cathepsin D maturation (Furuya et al., 2005). Beclin-1 can shuttle between the cytoplasm and the nucleus, although the role of the shuttling is unknown (Liang et al., 2001). These data together suggest an essential role for Beclin-1, possibly through protein interactions with hVps34, or changes in its kinase activity, in the induction of autophagy.

In 2006, UVRAG was the first component of the Beclin-1/Vps34 complex to be identified as a positive mediator of autophagy (Liang et al., 2006). UVRAG is a novel coiled-coil UV irradiation resistance-associated gene and is monoallelically mutated at 2001; Zeng et al., 2006).
high frequency in human colon cancer cells compared with 293T, NIH3T3, and MCF7 cells (Liang et al., 2006). In this study, hVps34 was only able to interact with UVRAG in the presence of Beclin-1, indicating Beclin-1 serves as a platform for the formation of UVRAG-hVps34-Beclin-1. Under conditions of starvation in colon and breast carcinoma cells, over-expression of UVRAG resulted in increased autophagosome formation and reduced clonogenicity. In response to nutrient starvation, Beclin-1 stimulates binding to another autophagy-associated protein, Bif-1 (Takahashi et al., 2007; Takahashi et al., 2008). Bif-1 is also known as Endophilin B1, and was originally discovered as a Bax-binding protein (Cuddeback et al., 2001). Bif-1 interacts with Beclin-1 through UVRAG and functions as a positive mediator of hVps34 activity. Bif-1 plays a role in autophagosome formation as well by colocalizing to autophagosomal membranes with autophagy proteins Atg5 and LC3 (Takahashi et al., 2007). The mammalian homolog to yeast Atg14, Atg14L/Barkor, was also identified by two independent groups as a component of the Beclin-1 autophagy complex (Matsunaga et al., 2009; Sun et al., 2008). Knockdown of Barkor gene expression by RNA interference compromised starvation- and rapamycin-induced autophagosome formation. Conversely, overexpression of Barkor leads to autophagy activation and increased number of autophagosomes.

A large complex of proteins, Beclin-1-Vps34-p150-UVRAG-Bif-1-Atg14L/Barkor, and Ambra1 are favorable mediators of autophagy. The interaction of Beclin-1 and Bcl-2 outside of this complex is thought to inhibit the proautophagic activity of Beclin-1 (Pattingre et al., 2005). In HT-29 cells, stable transfection of Bcl-2 inhibits starvation-induced autophagy by decreasing the association of Beclin-1 and hVps34, and reducing
the Beclin-1 associated PI3K activity of hVp34 (Pattingre and Levine, 2006). This finding suggests that Bcl-2 blocks the formation of the autophagy-promoting Beclin-1/hVps34 complex.

However, not all components of this large complex are mutually exclusive to each other. Activating molecule in Beclin-1-regulated autophagy, or Ambra1, was found to regulate autophagy and development of the nervous system. Ambra1 is involved in autophagy in controlling protein turnover during neuronal development, and in regulating normal cell survival and proliferation (Fimia et al., 2007). Itakura et al. found that Atg14 exists in a complex that consists of Vps34, Beclin-1 and p150, but lacks UVRAG (Itakura and Mizushima, 2009). This core complex localizes to the isolation membrane during starvation and is essential for autophagosome formation. In contrast, UVRAG was found to primarily localize to late endosomes. In yeast, the Class III PI3K Vps34 regulates membrane trafficking in two separate and distinct complexes: complex I (Vps34, Vps15, Vps30/Atg6, and Atg14) is involved in autophagy and complex II (Vps34, Vps15, Vps30/Atg6, and Vps38) functions in vacuolar protein sorting pathway (Abeliovich and Klionsky, 2001; Herman and Emr, 1990; Stack et al., 1993). In mammalian cells, the counterparts of Vps34, Vps15, and Vps30/Atg6 have been identified as hVps34, p150, and Beclin-1, respectively, and are evolutionarily conserved as components of the autophagy pathway. Beclin-1 was not found to be a part of the complex regulating endosomal trafficking (Figure 1-3) (Liang et al., 1998; Panaretou et al., 1997; Volinia et al., 1995; Zeng et al., 2006). While both Atg14 and Vps38 contain coiled-coil domains and act as a bridge between the lipid kinase Vps34 and Beclin-1, orthologs of mammalian
Atg14 and Vps38 had yet to be identified. Itakura et al. may have identified putative homologs of Atg14 and Vps38 based on a position specific iterative (PSI)-BLAST search.
of the National Center for Biotechnology Information (NCBI) database comparison of *S. cerivisiae* Atg14 (Itakura et al., 2008). Barkor was identified in both the mouse and human database with 13.1% identity and 37.2% similarity to yeast Atg14. The Vps38 candidate was found to have 10.2% identity and 31.9% similarity to UVRAG. Both human Atg14 and UVRAG interact with Beclin-1 and hVps34; however, Atg14 and UVRAG are not present in the same complex. While Atg14 is present on autophagic isolation membranes, UVRAG primarily associates with Rab9-positive endosomes. As UVRAG interacts with the hVps34-Beclin-1 complex and affects both processes of autophagy and endosome trafficking, it is hypothesized that UVRAG may combine the two activities of Atg14 and Vps38 into one protein.

Another protein called Rubicon was discovered as a negative mediator of the autophagy core complex. Whereas the interaction of Atg14L with Beclin-1 and hVps34 enhances lipid kinase activity and upregulates autophagy, Rubicon (RUN domain and cysteine-rich domain containing, Beclin 1-interacting protein) was found to reduce hVps34 activity and downregulate autophagy (Zhong et al., 2009a). The interaction of hVps34-Rubicon inhibits autophagosome formation. A summary of the roles of core complex subunits can be found in Figure 1-4.

In addition to regulation of autophagy by the PI3K complex, another complex consisting of several autophagy (Atg) proteins is also required for the formation and expansion of autophagosomes: It consists of a ubiquitin-like protein conjugation system containing Atg8-phosphatidylethanolamine (PE), Atg7, Atg10, and Atg12-Atg5. Atg12-Atg5
conjugation is essential for the formation of preautophagosomes, whereas Atg8 modification is essential for the formation of autophagosomes as well as autophagosome closure (Kabeya et al., 2000; Noda et al., 2009). These Ub-like conjugation pathways were identified as essential for proper formation of autophagic vesicles (Ohsumi and Mizushima, 2004). These pathways employ two Ub-fold proteins, Atg8 and Atg12, which become conjugated to their respective targets, the lipid phosphatidylethanolamine (PE) and the Atg5 protein, through an ATP-dependent reaction. The common E1-
activating enzyme Atg7, which couples ATP hydrolysis to form Atg8-Atg7 and Atg12-
Atg7 intermediates, first activates both Atg8 and Atg5. Activated Atg8 and Atg12 are
then donated by transesterification to their respective conjugating enzymes (or E2s), Atg3
and Atg10, which then form covalent adducts with the targets via an amide bond between
the C-terminal glycines of Atg8 and Atg12 and the ethanolamine moiety of PE and a
specific lysine residue in Atg5, respectively (Ichimura et al., 2000; Mizushima et al.,
1998). For Atg8, this glycine becomes exposed after processing of the initial translation
product by the Atg4 protease that removes the amino acids C-terminal to this residue
(Kirisako et al., 2000). It has been found that microtubules are required for the
autophagosome-lysosome fusion (Aplin et al., 1992). The microtubule-associated protein
light chain 3 (MAP-LC3) was originally identified by co-purification of microtubule
associated proteins 1A and 1B (Mann and Hammarback, 1994). MAP-LC3 is a
mammalian ortholog of Atg8. Interestingly, autophagosome formation is partially
inhibited by drugs that disturb actin polymerization, suggesting that actin filaments may
have a role in this process (Aplin et al., 1992; Blankson et al., 1995; Reggiori et al.,
2005). Furthermore, it has recently been shown that microtubules facilitate the formation
of autophagosomes, are required for fusion with endosomes, and are needed for
movement of autophagosomes and/or lysosomal vesicles (Kochl et al., 2006). Finally, a
group of regulatory proteins consists of members of the Atg family that are required for
disassembly of ATG complexes from autophagosomes (Atg2, Atg9, Atg18) (Levine and
Yuan, 2005).
THE ROLE OF PHOSPHATIDYLINOSITOL 3-KINASES (PI3K) IN AUTOPHAGY

Phosphoinositides (PI) are important regulators of many cellular functions including the recruitment of signaling proteins to membranes by direct interaction with phosphoinositide binding proteins. Site-specific phosphorylation of the inositol ring is an important mechanism for targeting these signaling proteins to specific membrane domains which are involved in the regulation of endosome to lysosome transport, endosome to Golgi sorting, and autophagy (Lindmo and Stenmark, 2006).

Phosphatidylinositol-3 kinases (PI3K) are enzymes that phosphorylate phosphatidylinositols (PtdIns) or PIs at the 3-position of the inositol ring. The phosphorylation of phosphatidylinositol lipids at the 3-position of the inositol ring by Class I PI3Ks in response to cell stimulation by growth factors and hormones leads to cell growth, cell cycle entry, cell migration, and cell survival. Hyperactivation of PI3K pathways has been linked to many diseases, including cancer formation and metastasis.

There are three classes of PI3Ks in mammalian cells. Class I PI3K use phosphatidylinositol (4,5)-biphosphate as their main substrate to produce phosphatidylinositol (3,4,5)-triphosphate. Class I PI3Ks are activated primarily through receptor tyrosine kinases (RTKs), G-protein-coupled-receptors (GPCRs), and the Ras oncogene and act primarily at the plasma membrane. Class II PI3Ks use PtdIns as their substrate yielding phosphatidylinositol 3-phosphate (PI3P). Similarly to Class I PI3Ks, Class II PI3Ks are activated by exogenous growth factors or hormones but also are involved in clathrin-mediated endocytosis (Gaidarov et al., 2001). In contrast, a single gene, hVps34, represents Class III PI3K and like other Class II PI3K, hVps34 uses PtdIns as its substrate yielding PI3P. hVps34 is constitutively active but is stimulated by amino
acid rich medium (Byfield et al., 2005). Whereas the Class III PI3K is required for autophagy, the oncogenic Class I PI3K/Akt pathway inhibits it, possibly through mTOR. Insulin, which is an activator of the Class I pathway through RTKs, inhibits autophagy as does constitutively active AKT expression (Lumeng and Saltiel, 2006; Petiot et al., 2000). Alternately, the tumor suppressor PI-3,4,5-P3-phosphatase PTEN can stimulate autophagy when overexpressed (Arico et al., 2001).

PI3P, the product of Class II and Class III PI3Ks, has several known effector proteins that contain a zinc finger FYVE domain named for known PI3P interacting proteins: Fab1, YOTB, Vac1, and EEA1. Similarly to FYVE domains, proteins containing pleckstrin-homology (PH) domains as well as Phox-homology (PX) domains can bind to PI3P. All three of these domains can be used as probes for PIs. Particularly, the 2x FYVE probe has been used to show that PI3P is found on the membranes of endosomes and autophagosomes (Gillooly et al., 2000; Vieira et al., 2001). Most proteins that contain FYVE or PX domains serve as regulators of endocytic membrane trafficking, whereas others function as regulators of autophagosome development and signal transduction (Stenmark and Gillooly, 2001). These findings lead to the hypothesis that hVps34 is an integral protein involved in the recruitment of proteins essential in the formation of autophagosomes. Indeed, Vieira et al. have shown that hVps34 and its product PI3P are essential in the autolysosome fusion, but how this enzyme does so remains to be elucidated (Vieira et al., 2001). While hVps34 is a cytosolic protein, its autophagosomal membrane association and activation require its interaction with the myristoylated serine/threonine adaptor protein kinase p150 (Panaretou et al., 1997). Overexpression of
p150 stimulates both the synthesis of PI3P by enhancing the kinase activity of hVps34 as well as autophagosome sequestration, suggesting that p150 is a major player as both a regulatory subunit of hVps34 and either directly or indirectly in autophagy induction (Petiot et al., 2000). Additionally, the hVps34/p150 complex was found to interact with the GTPases Rab5 and Rab7, both of which are well known regulators of endosomal trafficking and protein sorting (Stein et al., 2005; Vieira et al., 2003).
1.3 Materials and Methods

*Cell Culture and Reagents* – Dulbecco’s Minimal Essential Medium (DMEM) was obtained from Invitrogen. Fetal Bovine Serum (FBS) and tetracycline-free FBS were obtained from JR Scientific, Inc. and Clontech, respectively. MCF-7 and U251 cells were obtained from the National Cancer Institute Frederick Cancer DCT Tumor Repository (Frederick, MD) and were maintained at 37°C in a 5% CO₂ / 95% air atmosphere in DMEM supplemented with 10% FBS, and passaged every three to four days.

*Generation of Plasmid Construct for Expression of FLAG-tagged Beclin-1* - The cDNA encoding Beclin-1 was developed by Dr. X. Zeng by PCR amplification using *Pfu* polymerase (Stratagene, La Jolla, CA) from a cDNA template, reverse transcribed from human embryonic kidney (HEK) 293 cell mRNA. The PCR product was cloned into pCMV5 and modified by addition of a 5’ sequence encoding the FLAG epitope (DYKDDDDK).

*Stable Transfection of FLAG-Beclin-1 into MCF-7 Tet-off Cells* - MCF-7 Tet-off cells were purchased from Clontech (Palo Alto, CA) and maintained in DMEM, supplemented with 10% FBS and 100 µg/ml G418. Dr. X. Zeng at the Medical College of Ohio developed MCF-7 Tet-off cells. These cells were transfected with the FLAG-Beclin-1 plasmid using Lipofectamine Plus according to manufacturer’s instructions. To facilitate clonal selection, cells were co-transfected with a pTK-Hyg vector (Clontech, Palo Alto,
Co-transfected clones resistant to 200 µg/ml hygromycin B (Clontech, Palo Alto, CA) were selected and tested for expression of FLAG-Beclin-1 by immunoblot analysis with mouse monoclonal anti-FLAG (Sigma, St. Louis, MO) in the presence or absence of 1µg/ml doxycycline hydrochloride (Dox, Sigma, St. Louis, MO). In the presence of Dox (+Dox), transcription of FLAG-Beclin-1 or EGFP is not induced; in the absence of Dox (-Dox), transcription of FLAG-Beclin-1 or EGFP is induced. Positive clones were maintained continuously under +Dox conditions until experiments were initiated. To turn on the expression of FLAG-Beclin-1, the cells were washed with Hanks balanced salt solution (HBSS) to remove residual Dox and incubated in the absence of Dox for 3 days.

Fractionation of cells to generate membrane and cytosol fractions – ZR75 cells were harvested by trypsinization and washed once in PBS before swelling in a 5x packed cell volume (PCV) of buffer containing 100 mM HEPES pH 8.0, 15 mM MgCl₂, 100 mM KCl, 100 mM DTT, and a protease inhibitor cocktail tablet (Roche). Cells were lysed with a 27G needle and centrifuged at 100,000 x g for 1 hour at 4°C. The resulting supernatant containing the cytosol was collected and stored after addition of 5x Laemmli sample buffer. The pellet containing membranes and organelles was resuspended in an equal volume of 1x Laemmli sample buffer. Calculation of insoluble to soluble band intensity was estimated by assigning Lane 1 of each immunoblot (nutrient rich cytosol, S100) a value of 1.
Immunoprecipitation of Endogenous Beclin-1 Protein Complexes - MCF-7 or U251 cells were grown to 80% confluence in 100-mm dishes in DMEM with 10% FBS. The cells were washed three times with HBSS, scraped from the dish and homogenized in IP buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, and protease inhibitor cocktail). The lysate was centrifuged at 10,000 x g for 15 min at 4°C and the supernatant solution was incubated with goat polyclonal IgG against Beclin-1 (2 hours at 4°C), followed by 1 hour incubation with protein A sepharose beads. The beads were washed three times with IP buffer, twice with phosphate-buffered saline (PBS), and then the immune complexes were eluted from the beads and subjected to SDS-PAGE and immunoblot analysis. Primary antibodies used for immunoblot analysis included mouse monoclonal against Beclin-1 (BD Biosciences, San Diego, CA), and rabbit polyclonal against hVps34 (Zymed Laboratories, South San Francisco, CA).

Immunoprecipitation of FLAG-Beclin-1 Protein Complexes Using Anti-FLAG M2 Affinity Gel - Anti-FLAG M2 affinity gel (mouse anti-FLAG beads) (Sigma, St. Louis, MO) is a purified murine IgG1 monoclonal antibody covalently attached to agarose by hydrazide linkage. To immunoprecipitate FLAG-Beclin-1 from MCF-7 Tet-off cells expressing FLAG-Beclin-1, cells were seeded in 100-mm dishes the day before the experiment, washed three times with HBSS, scraped from the dish and homogenized in IP buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, and protease inhibitor cocktail). The lysate was centrifuged at 10,000 x g for 15 min at 4°C and the supernatant solution was incubated with mouse anti-FLAG beads for 30 minutes at 4°C. The beads then were collected by centrifugation for 1 min at 1,000 x g and the
supernatants were removed by aspiration. The pellets were washed three times with IP buffer. After aspirating the final wash supernatants, bound proteins were eluted from the beads with 100 µl of 100 µg/ml FLAG peptide (Sigma-Aldrich, St. Louis, MO). The proteins were solubilized with 25 µl 5x SDS sample buffer. Aliquots of whole cell lysate (1/10 of the total volume before immunoprecipitation with mouse anti-FLAG beads) were subjected to SDS-PAGE and immunoblot analysis along with immunoprecipitates. Primary antibodies used for immunoblot analysis included rabbit polyclonal anti-FLAG (Sigma), rabbit polyclonal against hVps34 (Zymed Laboratories, South San Francisco, CA), and a rabbit polyclonal against p150/PI3KR4 (C-term) (Abgent, San Diego, CA).

Identification of FLAG-Beclin-1 associated proteins by Nano-LC-Tandem-MS - After immunoprecipitation of FLAG-Beclin-1 complexes in MCF-7 cells and MCF-7 FLAG-Beclin-1 cells was performed as described above, lysate was run on an SDS-PAGE and silver-stained using a mass spectrometry compatible silver-staining kit as instructed by manufacturer (Invitrogen). Silver-stained bands of interest were excised and trypsin digested with sequencing-grade, modified trypsin (Promega) overnight at 37°C. Dr. V. Basrur performed the following procedures in the University of Toledo proteomics core facility. Peptides were extracted with 60% acetonitrile: 0.1% TFA. Extract was concentrated down to ~ 15 µl using a vacufuge. Two µl of the sample was separated on a reverse phase column (75 µm id X 16 µm Aquasil C18 Picofrit column, New Objectives). Eluent (peptides) were directly introduced into ion-trap mass spectrometer (LCQ-Deca XP Plus, Finnigan) equipped with nano-spray source. Mass spectrometer was operated on a double play mode where the instrument was set to acquire a full MS scan (400-2000
m/z) and a collision induced dissociation (CID) spectrum on the most abundant ion from the full MS scan. CID spectra were either manually interpreted or searched against an appropriate non-redundant database using TurboSEQUEST.
1.4 Results

**Beclin-1 and hVps34 Co-elute in a Large 500-600kD Complex in MCF-7 Breast Cancer Cells and U251 Glioblastoma Cells**

Mammalian cells express a class III PI3K called hVps34, a known key regulator of autophagosome formation and that, in addition to Beclin-1, is essential for autophagy. Beclin-1 is an important protein in the regulation of autophagy, but the underlying molecular mechanism of Beclin-1 action in mammalian cells is unclear. Little is known about the molecular basis of its tumor suppressor activity; but because Beclin-1 is involved in autophagy and hVps34 interacts with Beclin-1, it is postulated that Beclin-1 tumor suppressor activity may be related to its interaction with hVps34. Thus, we hypothesize that Beclin-1 regulates autophagy and, by inference, type II programmed cell death (PCD) through its interaction with hVps34. Moreover, autophagosomes are not generated in cells treated with PI3K inhibitors, further indicating the importance of the phosphatidylinositol 3-kinase activity of hVps34. Defining the components of the Beclin-1/hVps34 complex can identify key regulatory steps in the autophagy pathway that could elucidate a mechanism of manipulating the autophagic cell survival response. Beclin-1 has been shown by several laboratories including ours to interact with hVps34 in mammalian cells (Furuya et al., 2005; Kihara et al., 2001; Zeng et al., 2006). However, we do not know whether there are other interacting proteins within the Beclin-1/hVps34 complex. To first address this question, the soluble fraction of MCF-7 human breast carcinoma cells was subjected to gel filtration chromatography (Zeng, 2005). Beclin-1 and hVps34 co-eluted in a broad peak with a molecular mass of 500-600kD.
Consistent with the observation in MCF-7 cells, the 60kD Beclin-1 also co-eluted with the 100kD hVps34 in U251 glioblastoma cells indicating that at least in the cytosol this large complex exists universally and consists of other proteins besides Beclin-1 and hVps34. Additionally, siRNA induced suppression of Beclin-1 in U251 cells caused hVps34 to shift to a fraction of 200-300kD, which suggests that suppression of Beclin-1 expression disrupts components of the hVps34 PI3K complex (Zeng, 2005; Zeng et al., 2006).

The Myristoylated Adapter Protein p150 Co-Immunoprecipitates with Beclin-1 and hVps34 in Beclin-1 Overexpressed MCF-7 Cells

To identify other proteins within the Beclin-1/hVps34 complex, we developed a MCF-7 human breast cancer cell model in which a tetracycline repressible promoter can express FLAG-tagged Beclin-1 in the absence of tetracycline. In the MCF-7 Tet-off system, Beclin-1 expression is turned off when tetracycline (Tc) or doxycycline (Dox; a Tc derivative) is added. There are two plasmids involved in this system. One is the pTet-off regulatory plasmid, which encodes a fusion protein TetR/VP16, a tetracycline-controlled transcriptional activator (tTA). The other is the response plasmid which expresses Beclin under control of the tetracycline-response element (TRE), located just upstream of the minimal CMV promoter (PminCMV). PminCMV lacks the strong enhancer elements normally associated with the CMV immediate early promoter. In the presence of doxycycline, tTA cannot bind the TRE on the response plasmid and the transcription of Beclin-1 is inactivated. Uninduced cells (+Dox) were used because even in the presence
of doxycycline, MCF-7 Tet-Off cells have low expression of FLAG-Beclin-1 (Figure 1-5). We do not know if overexpressed FLAG-Beclin-1 will non-specifically bind to non-interacting proteins, therefore we used a FLAG-tagged cell model that has expression levels more comparable to those of endogenous protein (+Dox). MCF-7 FLAG-tagged Beclin-1 cells were immunoprecipitated with anti-Flag M2 affinity gel, and subjected to SDS-PAGE to identify interacting proteins of interest by mass spectrometry. In Figure 1-6, immunoprecipitated MCF-7 FLAG-Beclin-1 whole cell lysate was compared to the lysate of wild type MCF-7 on a silver stained SDS-PAGE gel, and proteins of interest were trypsin digested and the resulting peptides analyzed by nanoelectrospray ionization tandem mass spectrometry (Nano-LC-Tandem-MS). Interestingly, the results of the mass spectrometry identified p150 coimmunoprecipitating with FLAG-Beclin-1 in addition to hVps34. These results were also confirmed by immunoblotting with hVps34 and p150.
antibodies (shown in Figure 1-10). Additionally, several other proteins were identified in the complex including Hsp70, PP2A inhibitor, and actin. Hsp70 was of initial interest because of its role in chaperone-mediated autophagy.
However, subsequent coimmunoprecipitations of endogenous Beclin-1 were unable to confirm the specific interaction between Hsp70 and Beclin-1, therefore coimmunoprecipitation of FLAG-Beclin-1 and Hsp70 may be an artifact of non-specific binding of the protein to the anti-FLAG conjugated beads (data not shown).

The Beclin-1, hVps34, and p150 Complex Resides in Both the Cytosolic and Membrane Fractions of MCF-7 Cells, and is Recruited to the Membrane after Nutrient Starvation

In the previous experiment MCF-7 cells were lysed in detergent, therefore we do not know the subcellular localization of the complex. MCF-7 wild-type cells and MCF-7 FLAG-Beclin-1 cells were fractionated into the S100 (cytosolic) and P100 (insoluble) fractions, immunoprecipitated for FLAG and immunoblotted with anti-FLAG, anti-hVps34, and anti-p150 antibodies. Surprisingly, p150 elutes in both the cytosolic and membrane fractions (Figure 1-7). These results indicate that membrane localization is most likely regulated by a protein other than p150. These results were similar to previous coimmunoprecipitation in our laboratory of endogenous Beclin-1 and hVps34 (Zeng et al., 2006). Additionally when cells were amino acid and serum deprived there is a shift of the Beclin-1/hVps3/p150 complex to the membrane fraction (Figure 1-8). This suggests a role for the Beclin-1/hVps34/p150 complex in producing PI3P through hVps34 kinase activity in autophagosome assembly under conditions of nutrient deprivation.
Figure 1-7: Beclin-1/hVps34/p150 Complex Resides in Both Cytosolic and Membrane Fractions. MCF-7 cells (control) and MCF-7 FLAG-Beclin-1 cells were fractionated into soluble (S100) and insoluble (P100) compartments after a 100,000 x g centrifugation. Lysates were then immunoprecipitated with mouse anti-FLAG affinity beads and immunoblots were performed for p150 (upper panel), hVps34 (middle panel) and FLAG (lower panel) to detect protein expression levels.
Figure 1-8: The Beclin-1/hVps34/p150 Complex is Recruited to the Membrane Fraction After Nutrient Starvation. MCF-7 FLAG Beclin-1 cells were starved of amino acids and FBS (-) or were treated with full growth media (+) for 4 hours. Cells were fractionated into soluble (S100) or insoluble (P100) compartments and immunoblotted with anti-p150, anti-hVps34, and anti-FLAG antibodies (A). The ratio of insoluble to soluble (P/S) hVps34 and p150 of nutrient deprived (-) or full media (+) treated cells was calculated as an approximate change in band intensity (B) from scans of the immunoblots.
Amino acids 80-107 of Beclin-1 are required for binding to p150, and significantly reduces binding of hVps34.

From the coimmunoprecipitation data above (Figure 1-7), we know that Beclin-1 interacts with both hVps34 and p150 but we do not know whether this interaction occurs through tethering of other proteins. A 27 amino acid FLAG-Beclin-1 deletion mutant (FLAG-Δ80-107-Beclin-1) originally created by Dr. Zeng in our laboratory as a Bcl-2 binding mutant was used to define the nature of the Beclin-1 protein complex interactions (Figure 1-9). Of note the Δ80-107 mutation does not include the coiled-coil domain of Beclin-1 or the evolutionarily conserved domain (ECD), which are required for gene expression and interaction with hVps34, respectively (Cao and Klionsky, 2007).

FLAG-Δ80-107-Beclin-1 was introduced into MCF-7 Tet-Off cells via the pTRE expression vector in the same manner as the FLAG-Beclin-1 construct to generate a stable cell line. In this experiment, MCF-7 wild-type cells, MCF-7 FLAG-Beclin-1 cells, and MCF-7-Δ80-107-Beclin-1 were immunoprecipitated for FLAG and immunoblotted.
with antibodies to FLAG, hVps34, and p150 in a similar manner as in the experiments above. To our surprise, the Δ80-107 Beclin-1 mutant designed to inhibit binding of Beclin-1 and Bcl-2, had reduced binding to hVps34 and p150 (Figure 1-10). This data confirms that amino acids 80-107 of Beclin-1 are important for the complex formation of Beclin-1 with p150 and hVps34.

Figure 1-10: Amino acids 80-107 of Beclin-1 are Required for Binding of Interacting Proteins Including p150 and hVps34. MCF-7 cells (control), MCF-7 FLAG Beclin-1 cells, or MCF-7 FLAG-Δ80-107-Beclin-1 cells were immunoprecipitated with anti-FLAG M2 agarose beads and immunoblotted with anti-p150 (upper panel), anti-hVps34 (middle panel), or anti-FLAG (lower panel) antibodies.
1.5 Discussion

To our knowledge, we are the first to identify p150 complexed with Beclin-1 and hVps34 in mammalian cells. However, because the MCF-7 cells used in our initial experiments were lysed in detergent, we did not know the subcellular localization of the complex. There are two possible hypothetical models: 1) p150 regulates the sub-cellular distribution of the complex where Beclin-1 and hVps34 are targeted from the cytosol to the myristoylated p150 at the autophagosomal membrane, or 2) the Beclin-1/hVps34/p150 complex resides in both the cytosol and membrane fractions. To test these models, MCF-7 wild-type cells and MCF-7 FLAG-Beclin-1 cells were fractionated, immunoprecipitated with anti-FLAG M2, and immunoblotted with anti-FLAG, anti-hVps34, and anti-p150 antibodies (Figure 1-7). Surprisingly, although myristoylation occurs either during or immediately after synthesis, p150 co-precipitated with hVps34 and Beclin-1 in both the cytosol and membrane fractions (Figure 1-7) (Farazi et al., 2001). Myristoylation is required for association of proteins with the plasma membrane however; there are reports of myristoylated proteins not associated with membranes (David-Pfeuty et al., 1993; Sefton and Buss, 1987). These results indicate that the second hypothesis is most likely correct, and membrane localization is most likely regulated by a protein other than p150. A likely candidate for this is the recently discovered Beclin-1 interaction protein Atg14L/Barkor, which was found to enhance the lipid kinase activity of hVps34 and is localized to nascent autophagosome membranes (Zhong et al., 2009a). Additionally when cells were nutrient- and serum-deprived there was a shift of the Beclin-1/hVps33/p150 complex to the membrane fraction, suggesting that an autophagic
stimulus recruits the complex to the autophagosomal membrane (Figure 1-8). This indicates a role for the Beclin-1/hVps34/p150 complex in producing PI3P through hVps34 kinase activity in autophagosome assembly. However because the cells were starved in Hank’s Balanced Salt Solution (HBSS) for 4 hours (as reported by Pattingre et al. to induce autophagic vacuoles in MCF-7 cells, (Pattingre et al., 2005)), we were unable to induce expression of the autophagy protein marker LC3-II in that time frame. This indicates that in this cell model starvation of 4 hours did not produce an autophagy end-point, although proteins necessary for autophagy (Beclin-1/hVps34/p150) were recruited to the membrane. Beclin-1 functions as a component of the hVps34 complex, and both proteins are critical proteins in autophagy. Studies have suggested that the Beclin-1/hVps34 complex cycles between the cytosol and membrane and is localized at the trans-Golgi network (TGN) (Kihara et al., 2001). Our laboratory found that Beclin-1 is not required for the proteolytic processing of procathepsin D from the trans-Golgi network to the lysosomes, suggesting that the localization of Beclin-1 at the TGN is not directly involved in vesicular trafficking (Zeng et al., 2006). Additionally, Beclin-1 does not colocalize with early or late endosomal markers. Taken together, Beclin-1-hVps34-p150 appears to function in engaging the autophagy pathway and not vesicular trafficking.

From the coimmunoprecipitation data, we know that Beclin-1 interacts with both hVps34 and p150 but we do not know whether there are other proteins in this complex. A 27 amino acid FLAG-Beclin-1 deletion mutant (FLAG-Δ80-107-Beclin-1) originally created by Dr. Zeng in our laboratory as a Bcl-2 binding mutant was used to define the nature of
the Beclin-1 protein complex interactions. In this experiment, MCF-7 wild-type cells, MCF-7 FLAG-Beclin-1 cells, and MCF-7-Δ80-107-Beclin-1 were immunoprecipitated with anti-FLAG M2 and immunoblotted with antibodies to FLAG, hVps34, and p150. Unexpectedly, the Δ80-107 Beclin-1 mutant had reduced association with hVps34 and reduced association with p150 below the detection level of the immunoblot. These data confirm that amino acids 80-107 of Beclin-1 is required for the interaction of Beclin-1 with p150, although this interaction may be mediated by other Beclin-1 complex proteins.

Generation of PI3P, the product of Class III PI3Ks, requires the catalytic activity of hVps34 and the regulatory activity of p150. Furuya et al. have demonstrated that a Beclin-1 mutation within the evolutionarily conserved domain spanning amino acids 244-337 is unable to enhance starvation-induced autophagy due to its inability to interact with hVps34 (Furuya et al., 2005). This binding mutant also has no Beclin-1 associated Vps34 kinase activity and lacks tumor suppressor function in an MCF-7 SCID mouse xenograft tumor model. These studies suggest that the interaction between these proteins may be an essential component of autophagy function. Subsequent publications in other laboratories found that Beclin-1 interacts with hVps34, p150, Bif-1, UVRAG, Rubicon, and Atg14L/Barkor (Liang et al., 2007; Sun et al., 2008; Takahashi et al., 2007; Zhong et al., 2009a). In yeast, Atg14 targets the Beclin-1-Vps34-Vps15 complex to the phagophore assembly site (PAS) and is required for localizing additional ATG proteins to the PAS. It was found that Atg14/Barkor is the protein responsible for recruiting the Beclin-1-hVps34-p150 complex to the autophagosome in mammalian cells (Zhong et al., 2009b).
Taken together, these results indicate that Beclin-1 is part of a large multi-protein complex that includes at least hVps34 and p150. The trimeric complex consisting of Beclin-1/hVps34/p150 resides in both the soluble and membrane cellular fractions; therefore, membrane targeting of Beclin-1/hVps34 is not due solely to the presence of the myristoylated p150. Results with the Beclin-1 Δ80-107 mutant indicate that Beclin-1 interacts either directly or indirectly with hVps34 and p150 within this large multimeric complex and that the Beclin-1/hVps34/p150 complex can be modulated by an autophagic stimulus.
1.6 Conclusions


2. Although myristoylation occurs either during or immediately after synthesis, p150 was found to associate with the Beclin-1 and hVps34 complex in both the cytosolic and membrane fractions of MCF-7 cells. This suggests that another yet unknown protein in this complex is responsible for translocation of the cytosolic complex to the autophagosomal membrane and based on reported literature, a plausible candidate is Atg14L/Barkor.

3. The Beclin-1/hVps34/p150 complex appears to be recruited to the membrane fraction after autophagic stimulation of MCF-7 cells.

4. An interaction between Beclin-1 is important for the interaction with p150, and occurs between amino acids 80-107 of Beclin-1, either directly or indirectly through tethering of p150 to other complex proteins, as evidenced by failure of a Beclin-1 construct with a deletion in this region to interact with hVps34/p150 in MCF-7 cells.
1.7 Summary

Beclin-1 and hVps34 co-elute together in a 500-600kD protein complex in MCF-7 breast cancer cells and U251 glioblastoma cells, indicating that Beclin-1 resides in a large multimeric complex consisting of proteins other than Beclin-1 and hVps34. The molecular mass of hVps34 in this large multimeric complex is reduced to 200-300kD when Beclin-1 expression is suppressed in U251 cells, suggesting that Beclin-1 is essential for the formation of the cytosolic hVps34 complex. Through FLAG-Beclin-1 overexpression, we found that Beclin-1 co-immunoprecipitates hVps34 and its myristoylated protein adapter p150 in MCF-7 breast cancer cells. Additionally, although myristoylation of proteins occur either during or almost immediately after synthesis, the Beclin-1-hVps34-p150 complex was found to reside in both the cytosolic and membrane fractions of MCF-7 cells, suggesting that another yet unknown protein in this complex is responsible for translocation of the cytosolic complex to the autophagosomal membrane. After an autophagic stimulation of MCF-7 cells, the Beclin-1-hVps34-p150 complex appears to be recruited to the membrane fraction, suggesting recruitment to the autophagosome. Subsequent publications in other laboratories found that Beclin-1 interacts with hVps34, p150, Bif-1, UVRAG, Rubicon, and Atg14L/Barkor, and that these proteins play key roles in recruitment of Beclin-1 and hVps34 to autophagosomal membranes (Liang et al., 2007; Sun et al., 2008; Takahashi et al., 2007; Zhong et al., 2009a).
Beclin-1 interacts with both hVps34 and p150 but we do not know whether this is a direct interaction or an indirect interaction through tethering of other autophagosomal membrane proteins. A 28 amino acid FLAG-Beclin-1 deletion mutant (FLAG-Δ80-107-Beclin-1) originally created by our laboratory as a Bcl-2 binding mutant was used to define the nature of the Beclin-1 protein complex interactions. FLAG-Δ80-107-Beclin-1 reduced interaction with the hVps34 complex and reduced association with p150 below the detection level of the immunoblot. These data suggest that amino acids 80-107 of Beclin-1 are required for interaction with p150, either directly, or indirectly through other protein interactions.

In summary, Beclin-1 is often part of a large multi-protein complex that includes at least hVps34 and p150. The trimeric complex consisting of Beclin-1/hVps34/p150 resides in both the soluble and membrane cellular fractions; therefore, membrane targeting of Beclin-1/hVps34 is not due solely to the presence of the myristoylated p150. Results with the Beclin-1 Δ80-107 mutant indicate that Beclin-1 associates with hVps34 and p150 either directly or indirectly through other complex proteins. Nutrient starvation results in translocation of the Beclin-1/hVps34/p150 complex from the cytosol to the membrane fraction, suggesting that the complex can be modulated by an autophagic stimulus.
Chapter 2

Mechanisms of Rottlerin-induced Autophagy and Cell Death in Breast Carcinoma Cells

2.1 Introduction

Rottlerin, a compound from Mallotus philippinensis, has been shown to induce growth arrest or apoptosis of human cancer cell lines (Lim et al., 2008; Soltoff, 2007). Initially, Rottlerin was reported to inhibit Protein Kinase C delta (PKCδ) ($IC_{50} = 3-6 \mu M$) 5- to 10-fold more potently than PKCα or PKCβ and 13- to 33-fold more potently than PKCε, ζ, or η. It also inhibited CAM kinase III at 3-6 μM, was inactive against SRC kinase, and was a weak inhibitor of PKA and casein kinase II (Gschwendt et al., 1994). At best, these potency differences of 5- to 13-fold are quite modest, pharmacologically speaking, and in practice would require careful manipulation of concentrations to produce meaningful differentiation of PKC isotype effects. In an extensive study of protein kinases and inhibitors, Rottlerin failed to show any PKC inhibitory activity at all against the α and δ PKC isotypes, even at 20 μM and very low ATP concentrations and under a variety of conditions (Soltoff, 2007). Thus, Rottlerin does not appear to have any value as a PKCδ-selective tool. Inhibition of PKC appears, at least in part, to be due to a
competition between Rottlerin and ATP (Soltoff, 2001). Furthermore, Rottlerin has been shown to be an uncoupler of mitochondrial oxidative phosphorylation (Kurosu et al., 2007; Soltoff, 2001). Rottlerin was also reported to increase the presence of autophagic vacuoles in the cytoplasm of pancreatic cells, membrane association of GFP-tagged LC3 to autophagosomes, and a marked induction of LC3-II protein, important hallmarks of autophagy (Akar et al., 2007). Additionally, inhibition of Beclin-1 expression by RNAi was shown to inhibit Rottlerin-induced autophagy (Akar et al., 2007). Therefore, we hypothesized that Beclin-1 is required for Rottlerin-induced LC3-II accumulation in breast cancer cells. The second portion of the dissertation aimed to explore the hypothesis that the cytotoxic effects of Rottlerin are related to its regulation of autophagy.
2.2 Literature

**ROTTLERIN**

Rottlerin (5,7-dihydroxy-2,2-dimethyl-6(2,4,6-trihydroxy-3-methyl-5-acetylbenzyl)-8-cinnamoyl-1,2-chromine), also called a mallotoxin, is a chemical compound derived from the plant *Mallotus philippinensis* (Figure 2-1). It was shown to have antiproliferative effects and induce apoptosis in various cell types by blocking protein kinase C (PKC) tyrosine phosphorylation (Lim et al., 2009; Soltoff, 2007; Song et al., 2008).

![Chemical Structure of Rottlerin](image)

*Figure 2-1: Chemical Structure of Rottlerin
Rottlerin is a phenolic ketone isolated from the plant *Mallotus philippinensis.*

To date, almost 500 studies have used Rottlerin, and most have employed it as a PKC delta (δ) selective inhibitor. To some extent Rottlerin was able to differentiate between PKC isoenzymes, with IC$_{50}$ values for PKCδ of 3-6 µM, PKCα, β, γ of 30-42 µM, and PKCζ, ζ, h of 80-100 µM (Gschwendt et al., 1994). Inhibition of PKC increases with increasing concentrations of ATP, suggesting that there is competition between Rottlerin and ATP. Among the protein kinases tested, Rottlerin suppresses only CaM-kinase III as
effectively as PKCd. The chemical structure of Rottlerin might serve as a basis for the development of novel inhibitors with improved selectivity for a distinct PKC isoenzyme, such as PKCd, or for CaM-kinase III. However, an increasing number of studies have demonstrated that Rottlerin might not act on PKCd directly but can produce intracellular changes that mimic those produced by direct inhibition of PKCd. Based on these findings for the non-specificity of Rottlerin on various protein kinases, actions of Rottlerin \textit{in vitro} appear to be independent of direct inhibition of PKCd activity. Instead, Soltoff et al. reported that Rottlerin has a direct effect on O$_2$ consumption that was unrelated to intracellular concentrations of Na$^+$ and K$^+$ (Soltoff, 2001). Their results suggested that Rottlerin might be acting as a mitochondrial uncoupler, and was similar to the effects of the mitochondrial uncoupler FCCP and its effect on increasing O$_2$ consumption in various cell lines. Notably in this study, as a consequence of uncoupled mitochondrial respiration from oxidative phosphorylation, Rottlerin significantly reduced cellular ATP levels. The decrease in ATP levels due to an increased consumption rate would have nonselective effects on kinase activity as well as other biological processes that require ATP. Cultured cells, including various cancer cell lines, rely on a combination of glycolysis and oxidative metabolism for ATP production and the contribution of oxidative and glycolytic production of ATP differs per cell line. For example, ATP production in human MCF-7 breast cancer cells was reported to be 80% oxidative and 20% glycolytic whereas the rat tumor cell line, PC12, relies more exclusively on glycolysis for ATP production (Guppy et al., 2002; Reynolds et al., 1982). In the PC12 study, perturbations in mitochondrial respiration due to uncoupling agents caused a delay in cell death. Tumors that have a large dependence on oxidative
metabolism could potentially be effectively inhibited by sub-toxic concentrations of Rottlerin, and this might prove to be an effective treatment in combination with chemotherapeutic agents.

Addressing the therapeutic potential of modulating cell death in cancer cells requires active chemicals with pharmacologically desirable properties. In addition to the anticarcinogenic properties of Rottlerin, early reports on the mechanism of action of Rottlerin found that treatment of metastatic cancer cell cultures with Rottlerin stimulated apoptosis and induced large vacuole-like structures at the ultrastructural level as well as by phase-contrast microscopy (Akar et al., 2007; Ohno et al., 2009). This led to an interesting question about whether autophagy or apoptosis occurs first in Rottlerin treated cells, and whether one is the preceding cause of the other, ultimately resulting in cell death. In a study that aimed to identify chemicals or pharmacological agents that modulate cancer cell death, four of 3,500 compounds were able to induce rapid autophagosome formation (Balgi et al., 2009). One of these was Rottlerin. Biochemical assays showed that the four compounds stimulated autophagy and inhibited the mammalian target of rapamycin Complex 1 (mTORC1) signaling in cells maintained in nutrient-rich conditions. mTORC1 is composed of mTOR, regulatory associated protein of mTOR (Raptor), mammalian LST8/G-protein β-subunit like protein (mLST8/GβL) and the recently identified partners PRAS40 and DEPTOR (Kim et al., 2002; Kim et al., 2003). The mTORC1 complex is characterized by the classic etiology of mTOR signaling by functioning as a nutrient, energy, and redox sensor, and by controlling protein synthesis (Kim et al., 2002). The activity of this complex is stimulated by insulin,
growth factors, amino acids, as well as oxidative stress (Kim et al., 2002). The compounds appeared to be specific to mTOR Complex 1, as they did not inhibit mTOR Complex 2, which also contains mTOR as a catalytic subunit, suggesting that they do not inhibit mTOR catalytic activity but rather inhibit signaling to mTORC1. The mTORC1 complex is negatively regulated by tuberous sclerosis complex 2 (TSC2). In complex with tuberous sclerosis complex 1 (TSC1), TSC2 inhibits the nutrient-mediated or growth factor-stimulated phosphorylation of p70S6K1 (S6K1) by negatively regulating mTORC1 signaling (Tee et al., 2002). S6K1 is a serine/threonine kinase that acts downstream of PIP3 and phosphoinositide-dependent kinase-1 (PDK1) in the PI3 kinase pathway and is a target substrate is the S6 ribosomal protein (Chung et al., 1994). Phosphorylation of S6 induces ribosomal protein synthesis. Rottlerin, unlike the other three compounds in this study, failed to inhibit mTORC1 signaling in tuberous sclerosis complex 2 (TSC2) -/- cells, suggesting that Rottlerin inhibits mTORC1 signaling by activating TSC2. If Rottlerin were a non-specific inhibitor that blocked the activation of any kinase due to depletion of ATP, it would also have an effect on the negative regulation of mTORC1 through TSC2. TSC2 can be phosphorylation by 5' AMP-activated protein kinase, or AMPK. AMPK is an enzyme that plays a role in cellular energy homeostasis and is required for translation regulation and cell size control in response to energy deprivation (Inoki et al., 2003). Inoki et al. discovered that depletion of cellular ATP by the glucose analog 2-deoxy-glucose (2DG), which blocks cellular glucose utilization by indirectly inhibiting hexokinase, the mitochondrial uncoupler FCCP, or Rottlerin, caused a dephosphorylation of S6K and an increase in the phosphorylation of AMPK and TSC2 function. This hyperphosphorylation of TSC2
protected cells against nutrient deprivation-induced apoptosis. Taken together, these results indicate that a mechanism of Rottlerin induced cell death may be phosphorylation of TSC2 by AMPK and inhibition of mTORC1 to protect cells against apoptosis. TSC2 activation instead induces autophagy, possibly as a cell survival mechanism that eventually cannot be overcome and results in cell death. Interestingly, another possible mechanism downstream of a signaling cascade in Rottlerin treated cells is upregulation of death receptor 5 (DR5) and stimulation of apoptotic cell death. Rottlerin significantly induced DR5 expression both at its messenger RNA and protein levels (Lim et al., 2009). This upregulation was dependent on binding to the transcription factor CCAAT/enhancer-binding protein-homologous protein (CHOP). Several journal articles have reported that ER stress-induced CHOP is a potential transcription factor for DR5 and that Rottlerin induces several ER-stress related proteins, including CHOP (Jung et al., 2006; Kim et al., 2008; Lim et al., 2008).

**PROGRAMMED CELL DEATH**

There are many avenues to cell death via intracellular signaling events or cell stress responses that can ultimately be defined based on morphological differences. Programmed cell death (PCD) is a genetically controlled mechanism of cell death, whereas necrosis traditionally has been regarded as a passive type of cell death. Type I cell death or apoptotic cell death is characterized by cell shrinkage, chromatin condensation, nucleosomal degradation and fragmentation, and is dependent on caspase activation. Autophagy is a caspase-independent form of programmed cell death, called Type II autophagic cell death. Many diseases have been reported to have deficiencies in
autophagy genes and/or alterations in the autophagic pathway, including neurodegenerative diseases and cancer. This is of interest to cancer therapy research because of recent findings that cancer cells undergo autophagy in response to cancer therapy treatments. This type of non-apoptotic cell death has been documented mainly by observing the formation of autophagic vacuoles over time in the cytoplasm of dying cells. There have also been several studies identifying apoptosis and autophagy occurring concurrently, inferring cross-talk between the two pathways (Boya et al., 2005; Gonzalez-Polo et al., 2005; Xie and Klionsky, 2007). Whether autophagy protects cells or causes cell death is controversial and depends on the cell type. It cannot be excluded that autophagy may function as an adaptive mechanism to allow cancer cells to withstand cancer treatments or nutrient deprivation. As noted above, it is interesting that autophagy shares several proteins in the oncogenic Class I PI3K pathway, possibly suggesting that a deregulation in the autophagy pathway can promote tumorigenesis.

**Apoptosis**

The process of Type I programmed cell death, or apoptosis, is generally characterized by distinct morphological events and energy-dependent biochemical mechanisms. Apoptosis is considered a vital component of various processes including normal cell turnover, proper development and functioning of the immune system, hormone-dependent atrophy, embryonic development and chemical-induced cell death. Excessive or insufficient apoptosis is a factor in many human diseases including neurodegenerative diseases, ischemic damage, autoimmune disorders and many types of cancer. The ability
to modulate the apoptotic potential of a cell is recognized for its immense therapeutic potential.

Apoptosis normally occurs during development and aging as a mechanism to remodel tissue cell populations. Apoptosis also may occur as a defense mechanism in response to infection or when cells are damaged due to disease or endogenous or exogenous DNA damaging agents (Norbury and Hickson, 2001). Although there are a wide variety of stimuli and conditions that stimulate apoptosis, both physiological and pathological, not all cells will die in response to the same stimulus. For example, chemotherapy results in DNA damage in some cells, which can lead to apoptotic death through a tumor suppressor p53-dependent pathway, whereas other cells are not susceptible due to an independence of p53 (Vazquez et al., 2008). There are two main apoptotic pathways in non-immune cells: the extrinsic death receptor pathway and the intrinsic mitochondrial pathway. These pathways can signal to each other, and molecules in one pathway can influence the other (Igney and Krammer, 2002). These pathways converge on the terminal execution pathway, which is initiated by cleavage of caspase-3, and results in morphological and molecular modifications such as DNA fragmentation, degradation of cytoskeletal and nuclear proteins, formation of apoptotic bodies, and cell shrinkage. Caspases are widely expressed in an inactive pro-enzyme form and once activated can often activate other pro-caspases, allowing initiation of a cascade of protease-mediated events. This proteolytic cascade, in which one caspase can activate other caspases, amplifies the apoptotic signaling pathway and leads to rapid cell death.
The intrinsic pathway that initiates apoptosis is non-receptor-mediated that produces signals through caspases, and these signals are mitochondrial-mediated events. Caspases have proteolytic activity and are able to cleave proteins at aspartic acid residues. Once caspases are initially activated there seems to be an irreversible commitment towards cell death. There are seven major caspases in the extrinsic and intrinsic pathways that have been identified and broadly categorized into initiators (caspase-2,-8,-9,-10), effectors or executioners (caspase-3,-6,-7) and inflammatory caspases (caspase-1,-4,-5) (Cohen, 1997). Apoptotic stimuli cause changes in the inner mitochondrial membrane resulting in the opening of the mitochondrial permeability transition pore, loss of mitochondrial membrane potential, and release of pro-apoptotic proteins normally sequestered in the intermembrane space. Pro- and anti-apoptotic Bcl-2 family proteins control the release of these mitochondrial proteins and the ratio of anti-apoptotic versus pro-apoptotic proteins regulates the degree of sensitivity to apoptosis. Overexpression of a pro-survival family member, or loss of a proapoptotic relative, can be oncogenic. The Bcl-2 family of proteins comprises both anti-apoptotic members including Bcl-2, Bcl-XL, and Mcl-1, and pro-apoptotic molecules such as Bax, Bak, and BH3-only domain molecules (Bim, Puma, and Bad). Once released into the cytosol, these mitochondrial proteins, including cytochrome c, (Smac)/direct IAP binding protein with low pI (DIABLO), and apoptosis-inducing factor (AIF), activate both caspase-dependent and –independent pathways (Saelens et al., 2004). P53 may play a critical role in the regulation of Bcl-2 family members, potentially through its translocation to the mitochondria (Li et al., 2004). It is thought that the main mechanism of action of the Bcl-2 family of proteins is the regulation of cytochrome c release from the mitochondria via alteration of mitochondrial
membrane permeability. The release of cytochrome c into the cytosol results in caspase-3 activation through formation of the cytochrome c/Apaf-1/caspase-9-containing apoptosome complex (Zou et al., 1999). The formation of this complex is the likely commitment step for cells to activate their caspases in response to stimuli that cause cytochrome c release. This complex is able to trigger characteristic features of cells dying through apoptotic cell death.

The extrinsic signaling pathways that initiate apoptosis involve transmembrane receptor-mediated interactions. These involve death receptors that are members of the tumor necrosis factor (TNF) receptor gene superfamily (Mahmood and Shukla, 2009). These death receptors share the cysteine-rich extracellular domains of TNF receptor genes, as well as a cytoplasmic “death domain” (Ashkenazi and Dixit, 1998). The best-characterized death receptors are CD95 (Fas/Apo1), TNFR1, and death receptor DR3, DR4, and DR5, also called Apo2 or TRAIL-R2. Upon respective ligand binding an adapter protein called Fas-associated death domain (FADD) is recruited to the death receptor and dimerizes pro-caspase-8, which in turn activates the downstream caspase-9, and commits the cell to apoptosis (Ashkenazi and Dixit, 1998).

The extrinsic and intrinsic pathways both converge at the executionary phase, where the activation of execution caspases 3, 6, and 7 activate cytoplasmic endonucleases, which degrade nuclear material, and proteases that degrade nuclear and cytoskeletal proteins. These executioner caspases cleave various substrates including but not limited to, cytokeratins, PARP, and α-fodrin (Salvesen and Dixit, 1999). These effector substrates
cause the morphological and biochemical features that are the hallmark of apoptosis as listed above.

Since apoptosis occurs via a complex signaling cascade, there are many points of opportunity to evaluate the activity of these proteins. Activators, effectors, and regulators of apoptosis can be assayed to detect which cells are undergoing apoptosis, if at all. Many features of apoptosis can overlap with necrosis and it is crucial to employ at least two of these biochemical assays to confirm that cell death is occurring via apoptosis. Based on methodology of detection, apoptosis assays can be classified into six major groups: 1) cytomorphological alterations, 2) DNA fragmentation (i.e., TUNEL), 3) detection of caspases, their cleaved substrates (i.e., PARP), regulators, and inhibitors (pan-caspase inhibitors such as z-VAD), 4) membrane perturbations, and 5) mitochondrial assays (i.e., cytochrome c release) (Elmore, 2007).

Necrosis

Necrotic cell death or necrosis is a form of cell death that lacks features of either apoptosis or autophagy. It is characterized by a gain in cell volume (oncosis), swelling of organelles, plasma membrane rupture, and subsequent loss of intracellular contents in response to external factors such as infections, toxins, or injury (Kroemer et al., 2009). Although necrosis has long been considered an uncontrolled and passive form of cell death, necrosis can occur in a regulated fashion involving a precise sequence of signals. For example, death domain receptors such as TNFR1 and FasR have been shown to induce necrosis in the presence of caspase inhibitors (Byun et al., 2006; Vercammen et
al., 1998). Generally, cells that die due to necrosis do not send the same chemical signals to immune cells in vivo that cells that die by apoptosis do. This prevents phagocytic clearance of necrotic cells, leading to a build-up of tissue and cell debris at the localized site of cell death. Necrosis is a relatively early event that typically begins with cell swelling, chromatin digestion, disruption of the plasma membrane and organelle membranes. Late necrosis is characterized by extensive DNA hydrolysis, vacuolation of the endoplasmic reticulum, organelle breakdown, and cell lysis. The release of intracellular contents after plasma membrane rupture is the cause of inflammation in necrosis. After the initial insult, the causative cascade of events that occurs is unclear. Several different phenomena that define necrosis are apparent, including mitochondrial uncoupling and production of reactive oxygen species, lysosomal changes including membrane permeabilization, nuclear changes including fragmentation of PARP1, lipid degradation (following the activation of phospholipases and sphingomyelinases), and increases in cytoplasmic Ca2+ that results in activation of calpains and cathepsins (Golstein and Kroemer, 2007). It is believed that the serine/kinase receptor interacting protein-1 (RIP1) plays a crucial role in necrotic cell death. Due to the absence of a consensus on the mechanism by which necrosis is induced, it is necessary to exercise caution in using biochemical assays to distinguish necrosis from other forms of cell death. Several assays should be employed, including, but not limited to, activation of calpains and cathepsins, decrease in cellular ATP levels, plasma membrane ruffling and rupture, RIP1 phosphorylation, ROS generation, and specific PARP1 cleavage (Kroemer et al., 2005).
Of particular interest is High Mobility Group Box 1 (HMGB1) release because of recent reports of its specificity to necrotic cell death. HMGB1 is a nuclear protein highly conserved in mammalian cells and is passively released by cells that have died by necrosis. Several receptors for HMGB1 exist, and upon binding HMGB1 these receptors trigger adaptive immunity and promote the migration and proliferation of cells (including stem cells) to repair damaged tissue (Raucci et al., 2007). Interestingly, cells that are undergoing apoptosis use this system to modify their chromatin so as to bind HMGB1, which in turn is not released. In addition to passive release of HMGB1 during post-necrotic cell death, many cell types have the ability when stressed to secrete HMGB1 actively via a dedicated pathway and thus produce a damage signal. HMGB1 does not have a secretion leader sequence and therefore must be released via a non-classical, vesicle-mediated secretory pathway (Gardella et al., 2002).

**Macroautophagy**

Degradation of intra-cellular proteins is mediated by two major mechanisms within eukaryotic cells. The first is the non-lysosomal ubiquitin-proteasome pathway in which target proteins are polyubiquitinated and subsequently recognized by the 26S proteosome, which degrades the proteins into short peptides. The second mechanism involves pathways for lysosomal protein degradation, endocytosis, and autophagy. Depending on how the delivery of the cytoplasmic material to the lysosomal lumen occurs, there are three alternate routes of autophagic activity: 1) macroautophagy, 2) microautophagy, and 3) chaperone-mediated autophagy (Dunn, 1994; Klionsky and Emr, 2000). Macroautophagy is a non-selective process in which proteins and organelles are
sequestered by the formation of a double membrane structure, which envelops the materials to be degraded into a vesicle called an autophagosome. The autophagosome then fuses with a lysosome whose hydrolytic enzymes degrade the materials. In microautophagy, the membrane of the lysosome invaginates to form an internal cellular vesicle, similar to autophagic vacuoles formed in autophagy. Chaperone-mediated autophagy involves the targeting of proteins with a particular peptide sequence (KFERQ) by the heat-shock protein Hsp70 and delivery to the lysosome via interaction with its receptor within the lysosomal membrane, lysosome-associated membrane protein (Lamp) 2a.

Macroautophagy, or autophagy, is stimulated by the induction of a cellular stress signal such as amino acid or growth factor starvation (Kelekar, 2005). Induction of autophagy is inhibited under nutrient-rich conditions and induced by starvation. Autophagy is characterized by the sequestration of bulk cytoplasm from the \textit{de novo} synthesis of a double membrane called a pre-autophagosomal structure (Noda et al., 2002), or possibly from the smooth endoplasmic reticulum (Dunn, 1990). In this process, the double membrane structure elongates and sequesters a portion of cytoplasm forming an autophagosome. Autophagosomes do not yet contain lysosomal membrane proteins or enzymes at this step, and they are not acidic (Dunn, 1990). In mammalian cells, autophagosomes next fuse with lysosomes, receiving lysosomal membrane proteins, enzymes, and proton pumps, from the lysosomal vesicles. The proteins engulfed within the cytoplasm are then degraded by lysosomal hydrolases and the degradation products are transported back to the cytoplasm where they are recycled. Post-fusion,
autophagosomes develop into late autolysosomes, which are acidic and contain lysosomal membrane proteins and enzymes. Experiments using quantitative immuno-electron microscopy confirms the presence of lysosomal membrane proteins and enzymes in late autophagic vacuoles (Eskelinen et al., 2002). Autophagy occurs at a low basal level in normal, healthy cells, and is upregulated in times of stress when cells need to generate nutrients and energy in the form of ATP, thereby promoting cell survival. Various signals can induce the sequestration of cytoplasm, and regulation of autophagy most likely occurs during the sequestration step. The current understanding of regulation of mammalian autophagy has been elucidated through inhibitor studies, implicating signal transduction pathways in stimulating autophagosome biogenesis.

Methods for monitoring autophagy have evolved as the understanding of the mechanism of autophagy and autophagosome formation has increased. There are three major methods for measuring autophagic activity: 1) morphological methods including electron microscopy and monodansylcadaverine (MDC) staining, 2) biochemical methods including measurement of bulk degradation of long lived proteins, and 3) identification of specific autophagy marker proteins (Mizushima, 2004). The most widely used and conventional method for identifying cells undergoing autophagy is electron microscopy. In fact, the morphology of autophagosomes was initially documented by this method, unfortunately it was subsequently noted that differentiation between autophagosomes and autolysosomes is difficult (Ashford and Porter, 1962). MDC is an autofluorescent dye that stains double membrane autophagosomes as well as acidic lysosomes, but not endosomes, under specific fixation conditions in mammalian cells (Biederbick et al.,
1995). Therefore, as such, it is not a specific marker for autophagosomes. Autophagy, as described above, is a process that degrades long-lived proteins versus the ubiquitin-proteasome pathway, which degrades short-lived proteins. Intuitively, measuring the degradation of long-lived proteins by radiolabeling cellular proteins can be used to measure autophagic activity. This method is often used for detecting autophagy but is argued to be non-specific because autophagy is not the only cellular method for proteolysis of long-lived proteins.

The most reliable method for monitoring autophagy to date is the biochemical molecular marker MAP-LC3 or LC3 (Figure 2-2). LC3 resides in the cytosol and undergoes ubiquitin-like post-translational modification in which the C-terminal region is cleaved, exposing a glycine residue, and is then called LC3-I. This processed form becomes conjugated to phosphatidylethanolamine (PE) where it associates with autophagosomal membranes. This form is called LC3-II. Therefore, the accumulation of LC3-II is indicative of increased autophagy. On an immunoblot, LC3-II migrates faster than LC3-I, and this mobility is assayed. The ratio of LC3-II to LC3-I correlates with autophagic activity (Kabeya et al., 2000). However, as Klionsky et al. have recently noted, accumulation of LC3-II may indicate that there is a problem in the fusion of autophagosomes and autolysosomes, and high levels may indicate a defect in the autophagic pathway (Klionsky, 2005). Conversely, low levels of LC3-II may not be indicative of low levels of autophagy. Therefore, when assaying LC3 as an autophagosomal marker, it may be necessary to determine if changes in LC3-II are related to defective lysosomal turnover instead of increased de novo autophagy by using
lysosomal inhibitors. LC3 expressed as a fusion protein with EGFP is also used as a marker for autophagosome localization. Experiments using this method are widely cited, which requires the use of a fluorescent microscope instead of an electron microscope. This method is also useful for observing autophagosome formation in real time.

Sequestosome 1 (SQSTM1 or p62) is a multifunctional protein that binds ubiquitin and regulates activation of the nuclear factor kappa-B (NF-kB) signaling pathway. The p62 protein is found within, and aids in the formation of, cellular inclusion bodies together
with polyubiquitinated proteins. It is also present in cytosolic protein aggregates that accumulate in various chronic, toxic, and degenerative diseases (Pankiv et al., 2007). These protein bodies and aggregates form in response to cellular stress, such as amino acid starvation, oxidative stress, accumulation of defective ribosomal products, or inhibition of autophagy (Clausen et al., 2010). P62 also binds to LC3 via an LC3 recognition sequence during autophagosome formation. In doing so, p62 is constantly and selectively degraded by the autophagy pathway and this turnover can be monitored by immunodetection (Bjorkoy et al., 2009; Ichimura et al., 2008). Conversely, a lack or inhibition of autophagy leads to accumulation of p62. Intriguingly, the formation of ubiquitin-positive aggregates induced by inhibition of the proteasome is greatly suppressed in p62-deficient cells suggesting that p62 is a general mediator of inclusion body formation. Taken together, p62 appears to be required for both the formation and the degradation of polyubiquitin-containing bodies by substrate recognition by the autophagy protein LC3.

CELLULAR STRESS RESPONSES
In normal cellular homeostasis, there is equilibrium between the net rate of cell growth and the net rate of cell death. Exposure to cellular stress causes this homeostasis to shift in the direction of cell death. Despite this, depending on the type and severity of the cellular stress response, if the stimulus does not proceed beyond a certain threshold the cell can activate a protective response to try to maintain the rate of cell survival. Conversely, if the stressor exceeds that threshold, stress signaling culminates in cell death.
One of the main pro-survival activities of cells, the heat shock response, was originally described as a biochemical response of cells to mild heat stress (i.e., elevations in temperature of 3-5°C above normal) (Lindquist, 1986). It has since been recognized that many stimuli can activate this response, including oxidative stress. One of the main cellular consequences of these stresses is protein damage leading to the aggregation of unfolded proteins. In order to counteract this, cells increase the expression of chaperone proteins called heat shock factors (HSFs) that help in the refolding of misfolded proteins and alleviate protein aggregation. HSF1 is maintained in a cytoplasmic monomeric form through interaction with heat shock protein 90 (Hsp90) and co-chaperones (Voellmy and Boellmann, 2007). When cells are exposed to stressful conditions, there is an accumulation of unfolded proteins that compete with HSF1 for Hsp90 binding. HSF1 is released from the complex and can then translocate to the nucleus and bind to the promoters of target genes, leading to the expression of heat shock proteins including Hsp27 and Hsp70.

Secretory and membrane proteins undergo post-translational processing in the Golgi apparatus and ER including: glycosylation (Golgi), disulfide bond formation and proper folding (ER). Exposure of cells to conditions such as glucose starvation, inhibition of protein glycosylation, and oxygen deprivation causes accumulation of unfolded proteins in the ER, resulting in ER stress. ER stress results in the activation of a mechanism termed the unfolded protein response (UPR) (Schroder and Kaufman, 2005). The UPR is generally transmitted through activation of ER resident proteins, most notably inositol-requiring protein-1 (IRE1), protein kinase RNA (PKR)-like ER kinase (PERK), and
activating transcription factor 6 (ATF6). UPR signaling promotes cell survival by balancing the protein load and folding capacity in the ER. However, if the protein load in the ER exceeds its folding capacity, or a defect in the UPR exists, cells may die with apoptotic features (ER stress-induced cell death). The caspase-12/caspase-4 pathway and C/EBP homologous protein (CHOP) and IRE1-JNK pathways have been identified as mechanisms that regulate this type of cell death (Malhotra and Kaufman, 2007; Wang et al., 1996). CHOP is a transcription factor that is induced downstream of PERK and ATF6 pathways. It triggers the ER stress-induced cell death in part by suppressing Bcl-2 expression and inducing Bim expression (Mizushima et al., 2010; Mori, 2010; Puthalakath et al., 2007). IRE1 participates in ER stress-induced cell death by activating JNK through the binding with ASK1 and Traf2 (Urano et al., 2000).

There are several methods to evaluate whether the UPR and related processes are activated. The ER luminal domains of PERK, IRE1, and ATF6 interact with the ER chaperone GRP78 (glucose-regulated protein); however, upon accumulation of unfolded proteins, GRP78 dissociates from these molecules, leading to their activation. Detecting autophosphorylation of IRE1 at serine 724, autophosphorylation of PERK at threonine 980, and proteolytic processing of full-length ATF6 can be used to monitor this activation.
2.3 Materials and Methods

*Cell Culture and Reagents* – Dulbecco’s Minimal Essential Medium (DMEM) and Hank’s Balanced Salt Solution (HBSS) were obtained from Invitrogen. Fetal Bovine Serum (FBS) and tetracycline-free FBS were obtained from JR Scientific, Inc. and Clontech, respectively. Parental ZR75 and MCF-7 cells were maintained in high glucose DMEM + 10% FBS at 37°C and passaged every three to four days. Where indicated, cells were treated with 6 µM Rottlerin (Calbiochem), 50 µM Z-VAD-fmk (Bachem), 500 nM Staurosporine (STS), 100 µM C2-Ceramide, lysosomal inhibitors E64d (10 µM) and Pepstatin A (10 µM), and cycloheximide (1 µg/ml).

*Measuring Cell Viability with CellTiter-Glo* - The CellTiter-Glo Luminescent Cell Viability Assay Kit (Promega) uses luciferase as the detection enzyme. The luciferase enzyme requires ATP to generate light; therefore metabolically active cells that are producing ATP as energy for cellular respiration generate luminescence that can be measured quantitatively. Five thousand ZR75 or MCF-7 cells per 100 µl were seeded in a black 96-well microplate in DMEM supplemented with 10% FBS and incubated at 37°C overnight. The following day, 6 µM Rottlerin or an equivalent volume of DMSO was added directly to the wells and cell growth was recorded every twenty-four hours post-treatment. Prior to addition of CellTiter-Glo substrate, the microplate was removed from the incubator and allowed to equilibrate at room temperature for 30 minutes. One hundred µl of CellTiter-Glo reagent was added to the 100 µl of cell culture medium and mixed for 2 minutes on an orbital shaker. Luminescence was recorded 10 minutes after
reagent addition using a SpectraMAX M2e microplate spectrophotometer (Molecular Devices, Sunnyvale, CA). Values represent the mean ± S.D. of 3 replicates for each sample set. The amount of ATP is directly proportional to the number of viable cells present in the culture.

Measuring Cell Viability with CellTiter-Fluor – The CellTiter-Fluor Cell Viability Assay is similar to CellTiter-Glo in that it is a non-lytic reagent used to measure cell viability. CellTiter-Fluor is a single reagent fluorescence assay that measures the relative number of live cells in culture by intra-cellular protease activity. Live-cell proteases are only active in intact viable cells and are measured using a fluorogenic, cell-permeant, peptide substrate (glycylphenylalanyl-aminofluorocoumarin; GF-AFC). The substrate enters intact cells where it is cleaved by the live-cell protease activity to generate a fluorescent signal proportional to the number of living cells. This live-cell protease becomes inactive upon loss of cell membrane integrity and leakage into the surrounding culture medium. The assay was performed as per manufacturer’s instructions (Promega, Madison, WI).

Soft Agar Colony-forming Assay - Colony formation by ZR75 and MCF-7 cells in soft agar is a common method to monitor anchorage-independent growth. Colonies can be visualized approximately 14 days after seeding 5 x 10^5 cells into a 6-well plate. Cells were grown in DMEM supplemented with 10% FBS and treated with 6 µM Rottlerin for 0, 6, 24, and 48 hours. Autoclaved and solidified 1.2% Agar (SeaPlaque, FMC, Bioproduct) was re-melted at 37°C to create the base and top layer of agar. To make the base agar, melted agar is mixed 1:1 with 2x DMEM medium plus 10% FBS to make a
0.6% final agar, and allowed to solidify for 30 min. Cells that were adherent post-treatment were manually counted, and \(5 \times 10^5\) cells were pelleted and resuspended in 1.2% melted agar plus 2x DMEM and 10% FBS to make the final top layer agar 0.3%. The cells are maintained in a humidified incubator at 37 °C with 5% CO\(_2\), and after 14 days stained with MTT (Sigma Chemical Corp.). Colonies were manually counted in each well.

Cytofluorometric analysis of mitochondrial membrane potential - JC-1 Mitochondrial Membrane Potential kit was purchased from Biotium. Five thousand ZR75 cells were seeded in black 96-well plates with 100 µl phenol red-free medium. Twenty-four hours after plating, the cells were treated with DMSO or 6 µM Rottlerin for 0, 1, 6, and 24 hours. After washing with PBS, cells were incubated in phenol-free culture medium containing 2 µg/ml of 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazol-carbocyanine iodide (JC-1, Molecular Probes) for 30 min. The dye was then removed and cells were washed three times with PBS. Fluorescence intensity was measured immediately by fluorescence spectrometry (SpectraMAX M2e, Molecular Devices, Sunnyvale, CA). For monomeric JC-1 (green), excitation = 485, emission = 525; for mitochondrial aggregated JC-1 (red), excitation = 535, emission = 590. The ratio of JC-1 aggregates in the mitochondria (red fluorescence) to JC-1 monomers in the cytosol (green fluorescence) was calculated to represent mitochondrial membrane potential (MMP). MMP is defined as a membrane potential across the inner membrane of mitochondria that is formed by the action of the enzymes of the electron transport chain.
Long-lived protein degradation – ZR75 cells were plated at a density of 500,000 cells per 60 mm dish in triplicate with an extra set of each sample to be used for immunoblotting. The cell cultures were radiolabeled with 0.2 µCi/ml [¹⁴C]-Valine (Amersham Pharmacia Biotechnology) for 24 hours followed by washing the cells 3x with PBS. Protein degradation was allowed to proceed with the addition of 10 mM cold Valine in 10% FBS/DMEM for two hours and the medium was discarded. After the two hour short chase, long-term chase medium (10 mM Valine in 10%FBS/DMEM) was added to turnover short-lived proteins with and without 6 µM Rottlerin to the radiolabeled dishes as well as the nonlabeled dishes for approximately 16 hours. At the end of the overnight incubation the radiolabeled cell cultures were scraped directly in the culture medium and precipitated with 10% trichloroacetic acid (TCA). Samples were centrifuged for 10 minutes at 600 x g, supernatant was removed, and Econofluor scintillation fluid was added to the supernatant and pellet separately. Degradation of long-lived proteins was analyzed using a scintillation counter and calculated as percent TCA soluble counts divided by total radioactive counts. Nonlabeled dishes were harvested to immunoblot for LC3-II accumulation.

Guava-Nexin Personal cell cytometry - Apoptosis was examined with a Guava-Nexin Kit containing Annexin-V-PE and 7-amino-actinomycin D (7-AAD) for staining of apoptotic and necrotic cells, respectively (Guava Technologies Inc., Hayward, CA). Briefly, 150,000 cells were plated in 6-well plates and allowed to adhere overnight. The following day cells were left untreated, or treated with 6 µM Rottlerin or 500 nM staurosporine for 24 hours. The cell culture medium was then removed and the cells
were washed 1x with PBS, trypsinized, and counted. The cells were washed 1x with annexin binding buffer and incubated 20 min on ice in 50 µl annexin binding buffer containing Annexin-V-PE and 7-AAD per manufacturer’s protocol. Following the incubation, 450 µl of Nexin buffer was added to each microcentrifuge tube of cells and analyzed on a Guava Personal Cytometer (Guava Technologies Inc., Hayward, CA).

**Immunofluorescence microscopy** - Cells were grown on laminin-coated glass coverslips, washed with Hanks balanced salt solution (HBSS), fixed in ice-cold methanol for 10 min, and blocked with 10% goat serum in PBS. To detect endogenous LC3, coverslips were incubated for 1 hour with an anti-LC3 rabbit polyclonal antibody (Abgent, San Diego, CA) diluted in PBS plus 10% goat serum, followed by a 1 hour incubation with goat anti-rabbit IgG conjugated with AlexaFluor-488 (Invitrogen, Carlsbad, CA, USA). Photomicrographs were taken with a Nikon Eclipse 800 fluorescent microscope equipped with a digital camera and ImagePro software (Media Cybernetics, Silver Spring, MD).

**Lentiviral-mediated RNA interference of ATG5 in ZR75 cells** – ZR75 cells were transduced with lentivirus carrying shRNA designed to knockdown ATG5 or enhanced green fluorescent protein (EGFP) using the Lentiviral RNAi Expression System (Systems Biosciences, Mountain View, CA), according to the manufacturer’s instructions. The shRNA sequences used were the following: ATG5 top strand,

5’GATCCGCAACTCTGGATGGGATTGCTTCCTGTCAGACAATCCCATCCAGTTGCTTTTTG-3’; ATG5 bottom strand, 5’-

AATCAAAAAAGCAACTCTGGATGGGATTGTCAGACAATCCCATCCAGA
GAGTTTGC-3’. These sequences contain a BamHI and EcoRI restriction site overhang sequence so that they can be directionally cloned into the pSIH-H1 vector after the shRNA sequences are annealed. Clones were screened after transformation for shRNA template inserts by PCR as per manufacturer’s instructions. The pSIH-H1 shRNA vector contains a puromycin resistance gene. To package lentivirus, this construct was triple co-transfected with a pPack-Rev, pPack-Gag, and pPack-VSVG plasmids into 293TN cells. 48 hours post-transfection of 293TN cells, the virus in the media was harvested and filtered through a 0.45 µm filter. Two ml of the filtrate was placed in a 60-mm cell culture dish containing 5 x 10^5 ZR75 cells in the presence of 4 µg/ml polybrene. Selection with puromycin (1 µg/ml) was performed 24 hours post-transduction and cells were kept under selection pressure until assayed for expression.

Lentiviral-mediated RNA interference of Beclin-1 in MCF-7 cells - MCF-7 cells were transduced with lentivirus containing shRNA sequences targeting Beclin-1. Five validated shRNA clones targeting human Beclin-1 were purchased from Sigma-Aldrich (MISSION shRNA Lentiviral Particles). Standard configuration includes 200 µl of shRNA lentiviral particles at 10^6 TU/ml. Of the 5 clones, 5’-CCGGCCCGTGGATGGAATGAGATTCTCGAGAATCTCATTCCACGGGTTTTTG-3’ generated the most efficient and robust knockdown of Beclin-1 post-puromycin selection in MCF-7 cells. Non-targeting shRNA was used as a control.

Retroviral-mediated RNA interference of Beclin-1 in ZR75 cells – To suppress Beclin-1 expression in ZR75 cells, an shRNA oligonucleotide was subcloned into
pSUPER.retro.puro vector (OligoEngine, Seattle, WA), a retroviral siRNA expression vector. The oligonucleotide sequence (20/21), which was used for successful siRNA interference with Beclin-1 expression, corresponded to nucleotides 1201-1219 (5’-GGCAAGAUUGAAGACACAG-3’) downstream of the transcription start site of beclin, followed by a 9-nucleotide non-complementary hairpin sequence (TATCTTGAC) and the reverse complement of the initial 19-nucleotide sequence. A control vector was constructed with a similar insert where the 19-nucleotide sequence had no homology to any known human gene sequence. Retrovirus was produced in 293 GPG packaging cells (Ory et al., 1996) maintained in DMEM plus 10% heat-inactivated FBS with 1 µg/ml puromycin, 300 µg/ml G418, and 2 µg/ml doxycycline. For transfection, the 293 GPG cells were seeded at \(1.2 \times 10^7\) cells/dish on 100-mm dishes in DMEM containing 10% heat inactivated FBS. Twenty-four hours later 293 GPG cells were transfected with the pSUPER.retro.puro constructs using Lipofectamine-Plus reagent (Invitrogen, Carlsbad, CA). Forty-eight and 72-h after transfection, the virus-enriched medium was collected and passed through a 0.45 µm filter. Transductions of the ZR75 cells were performed on two sequential days in the presence of 4 µg/ml polybrene. Twenty-four hours after the second infection, the cells were trypsinized and re-plated in selection medium containing 1 µg/ml puromycin. After a selection period of 6 days, the surviving cells were pooled and used for studies described in the following sections.

*Immunoblotting for HMGB1 detection* - Conditioned media from cells treated with either DMSO, 6 µM Rottlerin (Calbiochem), 500 nM Staurosporine (STS), or 1% H\(_2\)O\(_2\) were filtered through a 0.22 µm filter. Equal volumes of filtrate or equal protein
concentrations of whole cell lysate were fractioned by SDS-PAGE and transferred to a polyvinylidene difluoride membrane. Membranes were probed with a 1:100 dilution of purified mouse anti-HMGB1 (Santa Cruz Biotechnology, San Cruz, CA) or mouse monoclonal anti-α-tubulin (Sigma-Aldrich, St. Louis, MO). Bound antibody was detected using a 1:3,000 dilution of goat anti-mouse horseradish peroxidase-conjugated antibody, visualized by chemiluminescence, and viewed on a Fluorchem HD2 instrument (AlphaInnotech, San Leandro, CA).

*Immunoblot analysis* – Antibodies used for immunoblotting were obtained from the following sources: mouse monoclonal antibody against Beclin-1 (BD Biosciences, San Diego, CA), rabbit polyclonal antibody against hVps34 (Zymed Laboratories), anti-p150 (PI3KR4, C-term; Abgent, San Diego, CA), mouse anti-PARP1 (BD Pharmingen, San Jose, CA), JNK, phospho-JNK, BiP, rabbit anti-caspase 7, rabbit anti-ATG5-ATG12, anti-Lamin A/C (Cell Signaling Technology, Danvers, MA); CHOP (Santa Cruz Biotechnology, Santa Cruz, CA), mouse monoclonal anti-α-tubulin and mouse anti-GAPDH (Sigma, St. Louis, MO), mouse anti-Beclin-1and mouse anti-p62 (Abgent, San Diego, CA), and rabbit anti-LC3 (APG8b, N-Term; Abgent, San Diego, CA). Protein was quantified in cell lysates by colorimetric assay using the Bradford based Bio-Rad reagent (Bio-Rad, Inc., Hercules, CA, USA). SDS-PAGE and immunoblot analyses were performed as described previously (Zeng et al., 2006) using enhanced chemiluminescent (ECL) detection (GE Healthcare, Piscataway, NJ, USA). Chemiluminescent horseradish-peroxidase (HRP) immunoblot signals were quantified using an AlphaInnotech FluorChem HD2 imaging system.
2.4 Results

**Rottlerin Reduces Cell Viability as Measured by CellTiter-Glo ATP Assay in Breast Cancer Cells**

To investigate the antiproliferative response to Rottlerin in breast cancer cells, the estrogen receptor positive breast cancer cells ZR75 and MCF-7 were treated with 6 µM Rottlerin in a time course over three days and analyzed for metabolic activity using the CellTiter Glo luminescent assay system. This concentration was chosen based on literature reports of the cytotoxic effects of Rottlerin on human carcinoma cells (Gschwendt et al., 1994; Liao et al., 2005). This assay is a method of determining viable cells by measuring ATP production. As shown in the Figure 2-3, Rottlerin significantly

![Figure 2-3: Rottlerin Reduces Cell Viability as Measured by CellTiter-Glo ATP Assay in Breast Cancer Cells. ZR75 cells (A) or MCF-7 cells (B) were treated with 6 µM Rottlerin for 72 hours and assayed for metabolic activity over time with CellTiter-GLO. CellTiter-GLO measures intracellular ATP in healthy, metabolically active cells. Relative Luminescent Units (RLU). Error bars are S.D. of triplicate wells and significance determined by Student’s t-test (P < 0.01).](image-url)
inhibited the viability of both ZR75 and MCF7 cells as shown by reduced ATP production in response to Rottlerin within 24 hours and was sustained over 3 days ($P < 0.01$).

**Rottlerin Reduces Breast Cancer Cell Clonogenicity in Soft Agar**

For determination of in vitro clonogenicity, ZR75 and MCF-7 cells were treated with 6 µM Rottlerin for 0, 6, 24, or 48 hours. Post-treatment, 5000 viable cells, based on a manual count of adherent cells, were plated on top of a 2.5 ml base layer of 1.2% agarose gel in DMEM in a 6-well cell culture plate. Cells were layered above this base layer in semi-soft media containing 1.5 ml of 0.3% agarose in DMEM with 10% FBS. The cells were allowed to grow in this semi-soft medium in a 37°C humidified incubator for two weeks. After the two-week period, colonies were stained with MTT and manually counted using a scanned picture of each well. Error bars are standard deviation of the mean. Clonogenicity of the cell populations was reduced significantly after 24 hours of exposure to 6 µM Rottlerin (Figure 2-4, A and B) ($P < 0.05$).

**Rottlerin Induces the Apoptotic Response Within 48 Hours in ZR75 Breast Cancer Cells**

To determine if Rottlerin induces apoptosis of ZR75 cells, cells were treated with Rottlerin (6 µM) for 0, 6, 12, 24 or 48 hours. Apoptosis was examined by observation of caspase activation and cleavage of PARP1, which is often regarded as a hallmark event of apoptosis. Immunoblotting analysis revealed that Rottlerin induces the degradation of
endogenous 116 kD PARP1 as shown by the appearance of 85 kD fragments (Figure 2-5, upper panel), which became pronounced 48 hours after Rottlerin treatment. We also examined the cleavage of Caspase 7; an executioner caspase cleaved by upstream caspases 10 and 9 in response to an apoptotic stimulus (Walsh et al., 2008). Cleavage of Lamin A/C was also examined. Lamins A and C are intermediate filament type proteins which form major components of the nuclear cytoskeleton and caspase substrates, serving as a marker for caspase activation (Luthi and Martin, 2007). Similar results of cleavage were obtained as compared with that of PARP1 degradation (Figure 2-5, middle and lower panels). These results indicate that PARP1 cleavage and caspase activation is associated with Rottlerin-induced apoptosis in ZR75 cells.

Figure 2-4: Rottlerin Reduces Breast Cancer Cell Clonogenicity in Soft Agar. ZR75 cells (A) or MCF-7 cells (B) were treated with 6 μM Rottlerin for 0, 6, 24, and 48 hours and assayed for clonogenic potential in soft agar. After treatment with Rottlerin in 60-mm dishes, cells were trypsinized and plated on top of a bed of 0.6% agar at a density of 5000 cells/well in a 6-well plate in 0.3% agar. Error bars are S.D. of triplicate wells. Significance of each time point compared to the 0 hour was determined by Student’s t-test (*P < 0.05).
Rottlerin Induces Annexin-V Positive Staining after 48 Hours of Treatment in MCF-7 Breast Carcinoma Cells

To further confirm the apoptotic response, MCF-7 cells were treated with STS as a positive control, or 6 μM Rottlerin, and subjected to Annexin-V staining. Annexin-V is a calcium-dependent phospholipid-binding protein with high affinity for phosphatidylserine (PS) (van Engeland et al., 1998). PS is exposed on the outer membrane bilayer in the early phases of apoptotic cell death during which the cell

![Image of WB: PARP, Caspase 7, Lamin A/C Western Blots]

**Figure 2-5:** Rottlerin Induces the Apoptotic Response within 48 hours in ZR75 Breast Cancer Cells. ZR75 were treated in a time course with 6 μM Rottlerin. Cleavage of apoptotic proteins PARP (upper panel), Caspase 7 (middle panel), and Lamin A/C (lower panel) were examined by western blot. Staurosporine (STS) was used as a control.
membrane remains intact. The translocation of PS residues to the outer layer of the plasma membrane can then be detected with Annexin V-labeled with the fluorochrome PE. Cells are also double labeled with the vital dye 7-AAD which stains dead cells and cells with damaged membrane integrity. Therefore, on a dot plot, the lower right quadrant measures the percentage of cells in early apoptosis, and the upper right quadrant, cells in either late apoptosis or cells that have undergone necrotic cell death. The results of this experiment (Figure 2-6) show that similar to cells treated with staurosporine (STS), Rottlerin induces a 336% increase in total Annexin-V positive
staining (59.3%) compared to untreated cells (13.6%). Cells that were either undergoing late apoptosis or necrosis in response to Rottlerin increased 146% (21.9% versus 8.9%).

**Rottlerin Induces Cell Death in ZR75 Cells and Cannot Be Prevented by the Pan-Caspase Inhibitor Z-VAD-fmk**

Rottlerin induces the apoptotic response in parental ZR75 cells as shown by immunoblotting of PARP1, Caspase 7, and Lamins A/C (Figure 2-5). However, as shown in Figure 2-7, the caspase inhibitor Z-VAD-fmk did not abrogate the cytotoxic effects caused by 6 µM Rottlerin. Rottlerin clearly stimulates the cleavage of PARP1 (Figure 2-7, B), a hallmark of apoptosis. Pre-treatment with the caspase inhibitor Z-VAD-fmk significantly blocked Rottlerin-induced PARP1 cleavage, although it did not affect the ability of Rottlerin to impair cell viability (Figure 2-7, A). A pan-caspase inhibitor cannot block the cytotoxic effect of Rottlerin thus, although the caspase inhibitor prevented PARP1 cleavage, it could not prevent cell death. This indicates that the cells are dying by a mechanism other than apoptosis.

**Rottlerin Rapidly Induces Autophagy while the Apoptotic Response is Delayed in ZR75 Breast Cancer Cells**

To further explore the mechanisms of Rottlerin-induced cell death in ZR75 cells, cells were treated in both a time course and dose titration of Rottlerin, and an immunoblot was applied to assess the cleavage of PARP1 during the cell death process. In this experiment, ZR75 cells were treated with 0, 3, 6, and 10 µM Rottlerin for 24, 48, and 72 hours. As seen in Figure 2-8, upper panel, 6 µM Rottlerin induced the cleavage of
PARP1 after 48 hours of exposure but not at 24 hours. The expression of LC3 was examined by immunoblot using anti-LC3 Ab to determine if other modes of cell death are simultaneously stimulated in response to Rottlerin. An antibody to LC3 is used routinely in immunoblot assays to detect the amount of non-lipidated LC3-I and lipidated LC3-II. LC3-II directly correlates to the number of autophagosomes. Using identical conditions as for the PARP1 analysis, treatment of ZR75 cells with Rottlerin resulted in a dramatic increase in LC3-II (14 kD) by 24 hours, whereas LC3-I (16 kD) was not detected (Figure 2-7: Rottlerin Induces Cell Death in ZR75 Cells and Cannot be Prevented by the Pan-caspase Inhibitor Z- VAD-fmk. Evidence for a caspase-independent pathway for cell death in ZR75 cells. ZR75 cells were treated with the caspase inhibitor Z- VAD-fmk (100 μM) for 1 hour prior to treatment with or without 6 μM Rottlerin in a time course. Cell growth was analyzed over time with CellTiter-Glo (A). At the end of the experiment cells were harvested and analyzed by immunoblot for PARP cleavage (B). The arrow refers to the fragment of PARP that results from caspase cleavage.)
The LC3 antibody used in this immunoblot can detect LC3-I as well as LC3-II in some cell lines, therefore the absence of LC3-I is most likely due to either a lower sensitivity of the antibody for this form or the levels of LC3-I in ZR75 cells is below the limit of detection. This increase in the autophagy marker LC3-II can be

Figure 2-8: Rottlerin Rapidly Induces Autophagy while the Apoptotic Response is Delayed in ZR75 Breast Cancer Cells. Evidence for a caspase-independent pathway of cell death in ZR75 cells. ZR75 cells were treated in a time course with 0, 3, 6, and 10 μM Rottlerin. Cell lysates were analyzed over time with western blots for PARP cleavage (upper panel), the autophagy marker LC3-II (middle panel), and GAPDH as a loading control (lower panel). The arrow indicates the caspase-cleaved form of PARP.
observed as early as 6 hours of treatment 6 µM Rottlerin (Figure 2-12). This suggests that an early effect occurs on the autophagy pathway prior to induction of caspase activation.

**Breast Cancer Cells Treated with Rottlerin Accumulate a Higher Level of LC3-II than Cells Treated with the Classical Autophagy Inducer, C2-Ceramide**

Autophagy occurs as a normal physiological process to remove superfluous proteins and organelles, but it also has survival-oriented functions occurring under both basal conditions and conditions of stress, such as nutrient depletion. During autophagy, membrane lipids and proteins within autophagosomes are recycled to maintain needed levels of ATP production and protein synthesis, thereby promoting cell survival. Autophagic cell death is a morphological term derived from electron microscopic observations and denotes a form of cell death in which abundant autophagic vacuoles appear in the cytoplasm. However, this descriptive account tells us nothing about the pathophysiological significance of autophagy. It is still undetermined whether an abnormal increase in autophagic vacuoles is the primary cause of cell death in response to cell stress, or whether it reflects an abnormal function of the autophagic process. For example, accumulation of autophagic vacuoles can be caused by an impairment of digestion of the vacuolar contents because of lysosomal dysfunction. This abnormal function of “accumulation” of autophagic vacuoles may not be caused by an increase in autophagy but rather a block in the lysosomal fusion step. Such complications make it difficult to evaluate the actual pathophysiological significance of autophagy. To determine if the increase in LC3-II in Rottlerin treated cells was due to modulation of
autophagy, we next compared LC3 processing in Rottlerin treated cells versus the classical autophagy inducer C2-Ceramide. As shown in Figure 2-2, recruitment of LC3 to nascent autophagosomes involves its proteolytic cleavage and lipidation. This processing step yields a polypeptide (LC3-II) with increased electrophoretic mobility compared to non-lipidated LC3-I. When autophagosomes fuse with lysosomes LC3-II is degraded by lysosomal hydrolases. The LC3-II and LC3-I bands can therefore be considered as characteristic proteolytic intermediates in autophagy. When compared to the classical autophagy stimulator, C2-Ceramide, Rottlerin increases the amount of LC3-II 40-fold in ZR75 over control treated cells (DMSO), and 16-fold in MCF-7 compared to control treated cells (Figure 2-9, B and D). Comparatively, C2-Ceramide increases the amount of LC3-II approximately 5-fold compared to the DMSO control treatment in both cell lines (Figure 2-9, B and D). Although LC3-I can be detected in MCF-7 cell lysate, LC3-I was not detected in ZR75 cells; this is most likely due to the low affinity of the antibody for LC3-I compared to LC3-II. An important question is whether autophagy stimulation in response to Rottlerin is pro-survival in response to an apoptotic stimuli or a cause of cell death itself. Such a finding would represent a major advance in our knowledge of how the autophagic machinery orchestrates a coordinated, multifaceted approach to the elimination of an apoptotic stimulus.

**Rottlerin Increases Immunofluorescent Punctate Staining of LC3 in ZR75 cells**

Since the increase of in the amount of LC3-II in response to Rottlerin treatment in ZR75 cells may reflect accumulation of the autophagosomes in these cells, we examined this possibility using morphological analyses. On treatment with Rottlerin for 24 hours, ZR75
cells did not alter cell morphology in response to Rottlerin compared with DMSO cells by phase-contrast microscopy, i.e. rounding or spindling (data not shown). However, immunofluorescent staining with rabbit anti-LC3 Ab showed an increase in cytoplasmic puncta tethered with LC3 in 6 µM Rottlerin-treated ZR75 cells (Figure 2-10). This suggests that autophagosomes accumulate in Rottlerin-treated ZR75 cells.

Figure 2-9: Breast Cancer Cells Treated with Rottlerin Induces LC3-II Greater Than Classical Autophagy Inducer C2-Ceramide. ZR75 and MCF-7 cells were treated with DMSO (vehicle control), 6 µM Rottlerin, or 100 µM C2-Ceramide for 24 hours. Cell samples were analyzed by western blot for LC3-II or GAPDH (A and C). Normalization of LC3-II expression to GAPDH is shown for ZR75 cells (B) or MCF-7 cells (D).
Rottlerin Induces an Accumulation of LC3-II in the Presence of Lysosomal Inhibitors E64d and Pepstatin A

Autophagy is invariably associated with conversion of LC3 from the cytosolic LC3-I, to the autophagosome-associated LC3-II form. Increased levels of LC3-II were clearly detected in extracts of Rottlerin-treated ZR75 and MCF-7 cells (Figure 2-9). Immunofluorescence showed an increase in LC3-positive vacuoles in Rottlerin-treated ZR75 cells (Figure 2-10). Nevertheless, LC3-II accumulation could be due to an inhibition, rather than an induction, of autophagy. For example, a defect in the fusion step of autophagosomes and lysosomes, or a defect in lysosomal degradation, would result in LC3-II accumulation. To test the possibility that Rottlerin acts as an inhibitor of lysosomal degradation, we analyzed endogenous LC3-II levels in Rottlerin-treated ZR75 and MCF-7 cells in the presence of the lysosomal inhibitors, E64d and Pepstatin A.

Figure 2-10: Rottlerin Increases Immunofluorescent Punctate Staining of LC3 in ZR75 Cells. Immunofluorescence staining of LC3 in Rottlerin treated ZR75 cells. ZR75 cells were grown on coverslips and treated for 24 hours with DMSO (vehicle control) or 6 μM Rottlerin. The cells were fixed and permeabilized with methanol. A representative field of view is shown.
In a quantitative immunoblot of LC3-II, Rottlerin increases LC3-II levels compared to DMSO in ZR75 cells (Figure 2-11, A and C) and in MCF-7 treated cells (Figure 2-11, B and D). In ZR75 cells, Rottlerin leads to an 1100% increase in LC3-II compared to DMSO (Figure 2-11, C). For MCF-7 cells, this increase is 1600%. This percent increase in LC3-II represents the total amount of LC3-II stimulated by Rottlerin. But how much of this total LC3-II accumulation is due to autophagosome biogenesis and/or how much is due to an inhibition of lysosomal delivery and turnover? To examine this, cells were treated with lysosomal inhibitors with and without Rottlerin.

Treating cells with E64d and Pepstatin A for 24 hours results in a suppression of lysosomal turnover in both ZR75 and MCF-7 cells as shown by an increase in LC3-II compared with DMSO, as expected (Figure 2-11). When ZR75 and MCF-7 cells are co-treated with Rottlerin and inhibitors, there is an increase in accumulation of LC3-II compared to that of inhibitors alone (Figure 2-11, C and D). In ZR75 cells, Rottlerin leads to a 160% increase in LC3-II accumulated when the inhibitors suppress lysosomal degradation (Figure 2-11, C). In MCF-7 cells, Rottlerin leads to a 68% increase in LC3-II accumulated when lysosomal function is inhibited. This indicates that in both ZR75 and MCF-7 cells, Rottlerin has the ability to stimulate new autophagosome formation. However, this percent increase in LC3-II when Rottlerin is added to a system where lysosomal proteases are inhibited is much less than the percent increase in LC3-II when Rottlerin is added alone (160% versus 1100% in ZR75 cells, and 68% versus 1600% in MCF-7 cells). Taken together, these results suggest that while there is some increase in
autophagosome biogenesis in response to Rottlerin, the majority of LC3-II accumulated in response to Rottlerin is due to inhibition of lysosomal degradation.

Figure 2-11: Rottlerin Induces an Accumulation of LC3-II in the Presence of Lysosomal Inhibitors E64d and Pepstatin A. ZR75 and MCF-7 cells were treated for 24 hours in complete media (DMSO), 6 μM Rottlerin, and lysosomal inhibitors E64d (10 μM) and pepstatin A (10 μM) with and without 6 μM Rottlerin. Cells were lysed and analyzed for LC3-II and GAPDH expression by western blot (A and B). LC3 normalized to GAPDH of triplicate samples is shown for ZR75 cells (C) or MCF-7 cells (D) and analyzed for percent increase \(((B-A)/A)\times 100\). Immunoblots shown are representative of the triplicate samples.
**Accumulation of LC3-II in Response to Rottlerin is Due to Arrest in LC3-II**

**Turnover in ZR75 cells**

To determine whether Rottlerin directly affects the turnover rate of LC3-II, ZR75 cells were treated with Rottlerin for 6 hours to induce accumulation of LC3-II (Figure 2-12). In one case the 6 hour treatment with Rottlerin was followed by an additional 16 hour treatment with Rottlerin in the presence of cycloheximide (CHX), to block new protein synthesis. Under this condition, the amount of LC3-II remained constant compared to Rottlerin treatment alone. However, when the initial 6 hour treatment with Rottlerin was followed by simultaneous removal of Rottlerin with continued inhibition of protein synthesis with CHX, LC3-II returned to pre-stimulation levels. Replacement of Rottlerin with complete media also had the same effect. These studies indicate that Rottlerin has a major inhibitory effect on the turnover of LC3-II. The results also indicate that, at least after 6 hours, the effects of Rottlerin can be reversed upon removal of the drug.

**Rottlerin Induces p62 Accumulation in ZR75 cells**

The p62 protein, also called sequestosome 1 (SQSTM1), is a ubiquitin-binding scaffold protein that colocalizes with ubiquitinated protein aggregates. The protein is able to polymerize via an N-terminal PB1 domain and can interact with ubiquitinated proteins via the C-terminal UBA domain (Geetha and Wooten, 2002). p62/SQSTM1 has also been found to bind directly to LC3 and GABARAP (Pankiv et al., 2007). p62/SQSTM1 is itself degraded by autophagy and may serve to link ubiquitinated proteins to the autophagic machinery to enable their degradation in the lysosome. Since p62 accumulates when autophagy is inhibited, and decreased levels can be observed when
autophagy is induced, p62 may be used as a marker to study delivery and degradation of autophagosomal contents in the lysosome (Bjorkoy et al., 2009). In this experiment, ZR75 cells were treated with 6 μM Rottlerin for 0, 6, and 24 hours, and p62 expression monitored by immunoblot with an anti-p62 Ab. A 30% increase in p62 accumulation after 24 hours of Rottlerin exposure is illustrated in Figure 2-13, D. This demonstrates
that similar to LC3-II, degradation of p62 is blocked in response to Rottlerin and further confirms an impairment of autophagic turnover in response to Rottlerin.

Figure 2-13: Rottlerin Induces p62 Accumulation in ZR75 Cells. Complete media was added to ZR75 cells (untreated) or 6 μM Rottlerin for 6 and 24 hours. After 6 hours (A) or 24 hours (C) cells were lysed and analyzed for p62 and GAPDH expression by western blot. p62 normalized to GAPDH is shown for triplicate samples of the 6 hour treatment (B) and 24 hour treatment (D). Immunoblots shown are representative of the triplicate samples (* P = 0.03).
Rottlerin Does Not Alter the Rate of Long-Lived Protein Degradation in ZR75 Cells

Fusion of autophagosomes with lysosomes is important for completion of the autophagic pathway via degradation of cytoplasmic components. Among the cytoplasmic constituents targeted for autophagolysosome degradation are stable, long-lived proteins within the cell. A simple radiolabeled amino acid–based pulse chase assay can be used to assess the function of the autophagic pathway in cells by quantitatively monitoring the turnover of long-lived proteins during induction of autophagy or during its inhibition (Seglen and Bohley, 1992). Rottlerin and classical stimulators of autophagy were examined for their effects on proteolysis of long-lived proteins (Figure 2-14, A). In this experiment, proteins were labeled overnight with 0.2 μCi/ml L-[14C] valine. After three rinses with PBS, short-lived protein degradation was allowed to proceed with the addition of 10 mM unlabeled valine in 10% FBS/DMEM for two hours and the medium was discarded. Unlabeled valine (10 mM) and either DMSO, 6 μM Rottlerin, or 100 μM C2-Ceramide were added to the cells, and the chase was maintained for 24 hours to monitor long-lived protein degradation. Ten percent trichloroacetic acid (TCA) was then used to precipitate proteins and the radioactivity in the supernatant was measured. The [14C] valine released was calculated as the percentage of the radioactivity in the TCA-soluble supernatant to the total precipitable cell radioactivity. ZR75 cells were also seeded in 60-mm dishes in parallel for measurement of LC3-II accumulation and GAPDH expression (Figure 2-14, B). Whereas cells treated with the classical autophagy inducer, C2-Ceramide, showed a 60% increase in protein degradation, treatment of ZR75 cells with Rottlerin did not alter the rate of proteolysis (Figure 2-14, A). In parallel, an immunoblot of LC3 showed accumulation of LC3-II in Rottlerin treated cells whereas in
comparison, C₂-Ceramide had a smaller stimulatory effect on LC3-II in ZR75 cells (Figure 2-14, B). Taken together, these results demonstrate that although Rottlerin induces an increase in the amount of LC3-II, it does not increase the rate of long-lived protein degradation. If Rottlerin is blocking autophagosome fusion with lysosomes, why doesn’t Rottlerin decrease long-lived protein degradation? One possibility is that proteosome degradation is upregulated as a compensatory mechanism to degrade long-
lived proteins in response to macroautophagy inhibition. Therefore the time frame of
detection of proteolysis may need to be adjusted.

**Rottlerin Induces LC3-II Independent of Beclin-1 Expression in ZR75 and MCF-7 Breast Cancer Cells**

We have now established that Rottlerin is an antiproliferative agent that simultaneously
induces autophagy and blocks autophagosomal fusion with lysosomes. Rottlerin also
stimulates caspase activation, which pre-treatment with the pan-caspase inhibitor Z-
VAD-fmk cannot reverse. We have not however identified the mechanism of Rottlerin
induced cell death. To approach this, we first set out to determine whether inhibiting the
expression of an essential autophagy gene will inhibit the accumulation of LC3-II. One
of the central regulators of autophagy in mammalian cells is Beclin-1. Beclin-1 is a
component of the class III phosphatidylinositol 3-kinase (PI3KC3) complex, which also
contains a PI3K catalytic subunit and a regulatory subunit (p150). *Beclin 1* was
identified as a haploid insufficient tumor suppressor gene and is monoallelically deleted
in ovarian, breast, and prostate cancers (Qu et al., 2003). Heterozygous *Beclin 1*+− mice
have reduced autophagy activity and increased incidence of spontaneous tumors (Qu et
al., 2003). This evidence illustrates a role for Beclin-1 in autophagy and cancer
development. How the function of Beclin-1 is specifically directed toward
autophagosomes in mammalian cells has been discussed in Chapter 1 of this dissertation.
To examine whether Beclin-1 is required for the Rottlerin induced accumulation of
autophagosomes, we suppressed Beclin-1 expression by lentiviral-mediated RNAi. A
short-hairpin sequence targeting Beclin-1 in ZR75 and MCF-7 cells reduced Beclin-1
expression > 90% (Figure 2-15, upper panel). This suppression of Beclin-1 did not immediately appear to have a direct effect on LC3-II accumulation (Figure 2-15, middle panel), which would be consistent with our finding that the major effect of Rottlerin is on lysosomal turnover of autophagosomes, rather than initiation of autophagy. However we now know the necessity of assaying LC3-II accumulation in the absence and presence of
lysosomal inhibitors to measure the effect of an autophagy inducer on lysosomal degradation (Mizushima and Yoshimori, 2007).

Knockdown of Beclin-1 Expression Reduces LC3-II Accumulation in the Presence of Lysosomal Inhibitors in MCF-7 Breast Cancer Cells

Next, we assessed the levels of LC3-II in response to Rottlerin in MCF-7 control siRNA and Beclin-1 siRNA (Beclin-1 knockdown or KD) cells in the absence and presence of lysosomal inhibitors E64d and Pepstatin A. Cells were cultured for 24 hours in medium supplemented with a combination of lysosomal inhibitors with or without 6 µM Rottlerin (Figure 2-16). Treatment with inhibitors plus Rottlerin significantly induced LC3-II accumulation in MCF-7 control cells over inhibitor treatment alone by 55% (Figure 2-16, A). In contrast, in MCF-7 Beclin-1 KD cells, there was not a significant increase in LC3-II when cells were co-treated with Rottlerin and inhibitors, versus inhibitors alone (Figure 2-16, B). This demonstrates that Rottlerin can induce new autophagosome formation when protease inhibitors interfere with lysosomal degradation, and that this increase in new autophagosome formation can be prevented by the absence of Beclin-1 expression. These results corroborate evidence that Beclin-1 is required for induction of autophagy, but not for the Rottlerin-induced block in autophagosome fusion with lysosomes.
Impairment of ATG5 mediated Autophagy Blocks Rottlerin-Induced LC3-II Expression in ZR75 Breast Cancer Cells

Autophagy protein 5 is a protein that in humans is encoded by the \textit{ATG5} gene.

Conjugation of Atg12 to Atg5 occurs immediately after synthesis, where the carboxy-terminal glycine of Atg12 forms an isopeptide bond with the α-amino group of a lysine...
residue in Atg5 (Geng and Klionsky, 2008). This linkage is required for LC3 conjugation to phosphatidylethanolamine on autophagosomal membranes to occur. The Atg12-Atg5 and LC3 conjugation systems are interdependent and a disruption in one system has a direct negative effect on the autophagic process. Therefore, the absence of expression of ATG5 should not allow growth and expansion of autophagosomal membranes in breast cancer cells. To test this, ZR75 cells were stably transduced with shRNA targeting ATG5 to specifically knock down the intracellular levels of this protein. As shown in Figure 2-17, upper panel, there was a significant and near complete reduction in the protein levels of ATG5 (ATG5 KD) in ZR75 cells compared to ZR75 cells transduced with non-targeting shRNA (Control, C). Furthermore, transduction of ATG5 shRNA resulted in a significant decrease in the amount of LC3-II after Rottlerin treatment (Figure 2-17, middle panel).

Impairment of ATG5-Mediated Autophagy Does Not Protect ZR75 Breast Cancer Cells from Rottlerin Induced Cell Death

We have now established that there is a small but significant portion of LC3-II accumulation due to increased autophagosome biogenesis in Rottlerin treated breast cancer cells. To determine whether the this increase in autophagy leads to Rottlerin-induced Type II autophagic programmed cell death (PCD), the ATG5 knockdown (KD) cell lines were subjected to a Rottlerin time course. The ATG5 KD and Control siRNA cell lines were treated with a time course of DMSO (blue line) or 6 µM Rottlerin (purple line) to examine the effect of ATG5 expression on the growth and viability of ZR75 cells (Figure 2-18). Control siRNA Cells (A) and ATG5 KD cells (B) were analyzed over a 72
hour period for metabolic activity using CellTiter-Fluor assay. ATG5 expression did not have an effect on cell growth or viability compared to non-targeting control ZR75 cells (Control siRNA). Additionally, the ATG5 KD cells were not able to survive longer than Rottlerin treated control siRNA cells. This suggests that near complete abrogation of autophagy by ATG5 depletion does not rescue cells from Rottlerin induced cell death. Therefore the cells are not dying by Type II autophagic PCD.

Figure 2-17: Impairment of ATG5-mediated Autophagy Blocks Rottlerin-Induced LC3-II Expression in ZR75 Breast Cancer Cells. Expression of the ATG5 gene was suppressed by lentiviral-mediated RNAi in ZR75 cells. Control siRNA cells (C) and ATG5 knockdown cells (KD) were treated with DMSO or 6 μM Rottlerin to examine the effect of ATG5 expression on LC3-II accumulation. Cells were analyzed after 24 hours for ATG5, LC3-II, and GAPDH expression by immunoblot.
Figure 2-18: Impairment of ATG5-Mediated Autophagy Does Not Protect ZR75 Breast Cancer Cells from Rottlerin Induced Cell Death. Expression of the ATG5 gene was suppressed by lentiviral-mediated RNAi in ZR75 cells. In parallel, ZR75 cells were transduced with lentiviral-mediated RNAi targeting no human or mouse gene sequence for use as a control. The pooled cell lines were treated with a time course of DMSO (blue line) or 6 μM Rottlerin (purple line) to examine the effect of ATG5 expression on the growth and viability of ZR75 cells. Control siRNA Cells (A) and ATG5 knockdown (KD) cells (B) were analyzed over a 72 hour period for metabolic activity using CellTiter-Fluor. Error bars are the S.D. of triplicate wells.
Autophagosome Biogenesis Induced by Rottlerin is Dependent on PKCδ Expression

Because of earlier reported literature that Rottlerin is a specific inhibitor of PKCδ (Gschwendt et al., 1994; Keenan et al., 1997; Lu et al., 1997), we performed an experiment to establish whether PKC plays a role in Rottlerin-induced autophagy. Wild-type mouse embryonic fibroblasts (WT MEF) and PKCδ-/- MEF were treated with lysosomal inhibitors with and without Rottlerin to determine if PKCδ is required for Rottlerin-induced autophagy and/or inhibition of lysosomal degradation (Figure 2-19). WT MEF and PKCδ-/- MEF show a similar increase in total LC3-II with Rottlerin treatment. Specifically, a 300% increase is observed in LC3-II in Rottlerin treated WT MEF over DMSO, and a 340% increase is observed in LC3-II in Rottlerin treated PKCδ-/- MEF over DMSO control. This percent increase is the total amount of LC3-II formed when new autophagosomes are being made and degraded with Rottlerin treatment.

This experiment was also performed in the presence of lysosomal inhibitors (Figure 2-19). In WT MEF cells and PKCδ-/- MEF cells there is an increase in LC3-II accumulation when lysosomal degradation is inhibited, as expected. When Rottlerin is added to WT MEF cells that have impaired lysosomal degradation (due to addition of lysosomal protease inhibitors), there is a 1000% increase in LC3-II compared to inhibitors alone. Recall from Figure 2-2 that when degradation of cytoplasmic material is inhibited, stimulation of new autophagosome formation by an autophagic stimulus could result in accumulation of LC3-II above and beyond that of the stimuli alone because autophagosomes will continue to form and accumulate. As a result, the percent increase of LC3-II when a stimulus is added to cells where lysosomal degradation is inhibited (i.e.
protease inhibitors), would be greater than the percent increase of LC3-II between stimuli and control treated cells, because lysosomal turnover is not allowed to occur. The fact that the percent increase in LC3-II observed when Rottlerin and inhibitor are added together over inhibitors alone (1000%) is far greater than the percent increase in the total LC3-II formed by Rottlerin alone (300%) suggests that Rottlerin is not inhibiting lysosomal degradation in WT MEF cells, and is only forming new autophagosomes.

Figure 2-19: PKCδ Expression is Required for Autophagosome Biogenesis Induced by Rottlerin in Mouse Embryonic Fibroblast Cells. Wild-type MEF cells (A) PKCδ +/- MEF cells (B) were treated with DMSO (Control) or 6 μM Rottlerin for 16 hours. Cells were analyzed for LC3-II and GAPDH expression by western blot. Normalization of LC3 to GAPDH is shown for triplicate samples of Wild-type MEF (C) and PKCδ +/- MEF cells (D). Immunoblot shown is a representative of a triplicate sample.
In PKCδ -/- MEF cells, the addition of Rottlerin to cells that have impaired lysosomal function due to lysosomal protease inhibitors leads to a 240% increase in LC3-II compared to inhibitors alone. Unlike the WT MEF cells, this percent increase is less than the total percent increase in LC3-II caused by the addition of Rottlerin alone (340%). This suggests that while Rottlerin is inducing new autophagosome formation in PKCδ -/- MEF cells, there is LC3-II accumulating due to Rottlerin inhibiting lysosomal degradation (see Figure 2-11). Additionally, the percent increase of LC3-II in Rottlerin treated WT MEF and PKCδ -/- MEF cells over the DMSO control treatment are similar (300% and 340%, respectively). If the absence of PKCδ expression promotes the inhibitory effect of Rottlerin, yet the percent increase of LC3-II in Rottlerin treated cells is similar with and without PKCδ, then there must be fewer autophagosomes formed by Rottlerin in the PKCδ -/- MEF cells. Therefore, we conclude that PKCδ is required for Rottlerin-induced autophagosome biogenesis. These results suggest that Rottlerin induced autophagy is PKC-dependent. However, we believe it is important to consider the possibility that Rottlerin’s effects may be due to inhibition of kinases other than PKCδ (Soltoff, 2007).

Rottlerin Stimulates ER Stress Prior to the Induction of Apoptosis in ZR75 Breast Cancer Cells

We have now established that Rottlerin causes a block in autophagosome fusion with lysosomes and also stimulates autophagosome biogenesis within 6 hours, and the apoptotic response occurs within 48 hours of exposure. In eukaryotic cells, most proteins
are either synthesized on soluble ribosomes or on ribosomes attached to the ER. Misfolded cytosolic and nuclear proteins are typically tagged with ubiquitin and degraded via the proteasome. The accumulation of misfolded proteins in the ER induces the unfolded protein response (UPR), which results in the expression of chaperones and other proteins that act as folding catalysts (Wek and Cavener, 2007). Several studies have reported a link between autophagy and ER function. For example, a specific subgroup of secretory proteins is necessary for autophagosome formation but not for vesicle formation, implying a close relationship between autophagy and the secretory pathway (Ishihara et al., 2001). It has also been reported that ER stress stimulates autophagy (Kouroku et al., 2007; Sakaki and Kaufman, 2008). Our next goal was to determine whether ER stress could be an early response ultimately leading to autophagy and the apoptotic response. Immunoblots for the expression of ER stress proteins in response to Rottlerin were performed over 48 hours. BiP/Grp78 is an ER lumenal protein that functions as an ER chaperone protein; it triggers the UPR by retaining misfolded proteins in the ER. BiP expression increases between 24 and 48 hours (Figure 2-20, upper panel).

C/EBP homologous protein (CHOP) is another protein also activated by ER stress and has been implicated in ER stress-induced apoptosis. Interestingly, CHOP expression increases early, within 6 hours of Rottlerin treatment (Figure 2-20, middle panel). JNK is a MAPK that is responsive to stress stimuli, and misfolded proteins in the ER induce its phosphorylation. Calphostin C was used as a control for stimulation of JNK activity, and it can be clearly seen that there is no JNK activation in response to Rottlerin (Figure 2-20, lower panel).
Figure 2-20: Rottlerin Stimulates ER Stress Prior to the Induction of Apoptosis in ZR75 Breast Cancer Cells. ZR75 were treated with a 48 hour time course of 6 μM Rottlerin. Expression of ER stress proteins BiP (upper panel), CHOP (middle panel), and phosphorylation of JNK (lower panel) were examined by immunoblot. Calphostin C was used as a positive control for JNK phosphorylation.
Rottlerin Reduces Mitochondrial Membrane Potential (MMP) Prior to Inducing Apoptosis in ZR75 Breast Cancer Cells

Soltoff previously demonstrated that Rottlerin directly uncouples mitochondrial respiration from oxidative phosphorylation, because Rottlerin treatment of cells increased the rate of oxygen consumption and at the same time reduced intracellular levels of ATP (Soltoff, 2001). To explore the possibility that Rottlerin may enhance apoptosis through this mechanism, we next examined the effect of Rottlerin on mitochondrial membrane potential (MMP). ZR75 cells were treated with 6 µM Rottlerin for different time periods (0, 1, 6, and 24 hours) and then incubated for 30 min with 2 µg/ml JC-1 dye directly in culture medium. The cells were washed 3 times with PBS and analyzed for fluorescence using a SpectraMAX microplate spectrofluorometer. The ratio of JC-1 aggregates in the mitochondria (red fluorescence) to JC-1 monomers in the cytosol (green fluorescence) was calculated to represent MMP. As shown in Figure 2-21, 6 µM Rottlerin significantly reduced mitochondrial membrane potential (ΔΨm, an electrical potential required for mitochondrial function including ATP generation), rapidly and as early as 1 hour after treatment and continued to be significantly reduced for 24 hours after treatment (Figure 2-21).

Rottlerin Decreases Intracellular HMGB1 After 48 Hours of Exposure

ER stress-induced cell death has been shown to proceed primarily through apoptosis. However in cells deficient in apoptosis, autophagy was found to promote necrosis in response to ER-stress (Ullman et al., 2008). Since ER stress proteins BiP and CHOP are upregulated after 6 hours of Rottlerin treatment (Figure 2-20), we hypothesized that ER-
stress induced autophagy causes necrotic cell death. High Mobility Group Box Protein 1 (HMGB1) is a non-histone nuclear protein with dual function including chromatin structure and transcriptional regulation (Lange and Vasquez, 2009). As a nuclear protein, HMGB1 binds DNA. Outside the cell, HMGB1 serves as a cytokine. For its cytokine activities to be manifest, HMGB1 must leave the nucleus for transit into the extracellular milieu. Although this translocation process was identified originally in macrophages treated with LPS, necrotic cells also release this protein (El Gazzar, 2007). As such, HMGB1 has been implicated as a cause of inflammation secondary to necrotic cell death. Since HMGB1 interacts strongly with chromatin, we investigated whether apoptotic cells

![Graph showing the effect of Rottlerin on mitochondrial membrane potential (MMP) in ZR75 breast cancer cells.](image)

**Figure 2-21: Rottlerin Reduces Mitochondrial Membrane Potential (MMP) Prior to Inducing Apoptosis in ZR75 Breast Cancer Cells.** ZR75 cells were treated with 6 μM Rottlerin for different time periods (0, 1, 6, and 24 hours) and then incubated for 30 min with 2 mg/L JC-1 dye directly in culture medium. The cells were washed 3 times with PBS and analyzed for fluorescence using a SPECTRAMax microplate spectrofluorometer. The ratio of JC-1 aggregates in the mitochondria (red fluorescence) to JC-1 monomers in the cytosol (green fluorescence) was calculated to represent MMP. Error bars are the S.D. of triplicate wells. Percent decrease was calculated from each time point as ((B-A)/A)*100 and are significant compared to the DMSO control treatment (P < 0.003).
may also release HMGB1. To assess whether HMGB1 is released in response to Rottlerin, we treated ZR75 cells with DMSO, Rottlerin, STS (a known inducer of apoptosis), and H$_2$O$_2$ (a known inducer of necrosis) for 0, 24, and 48 hours and analyzed culture supernatants (media) or lysate (whole cell lysate, WCL). Fifty µl of filtered media from ZR75 cells treated with DMSO, Rottlerin, STS, and H$_2$O$_2$ were analyzed on a SDS-PAGE gel and immunoblotted for HMGB1 (Figure 2-22, upper panel). As shown in Figure 2-22, HMGB1 was present in the media of H$_2$O$_2$ treated cells in contrast to DMSO or Rottlerin treated cells. To verify that 1) HMGB1 was indeed released from whole cells and 2) that the antibody detected HMGB1 of the appropriate molecular weight, we compared an immunoblot of the WCL of cells treated with DMSO, Rottlerin, STS, and H$_2$O$_2$ for the indicated times. As expected, HMGB1 was completely absent from the lysate of H$_2$O$_2$ treated cells and the HMGB1 band detected in media from ZR75 cells treated with H$_2$O$_2$ was similar in size to the band from the whole cell lysates (Figure 2-22, lower panels). Although Rottlerin treated whole cell lysates have less HMGB1 (Figure 2-22, bottom left), none was detected in the media in response to Rottlerin. This suggests that Rottlerin may cause release of HMGB1 in conjunction with necrotic cell death, but it may just not be possible to detect lower levels of HMGB1 in the media by immunoblot analysis. Further studies confirming necrotic cell death need to be performed.
Figure 2-22: Rottlerin Decreases Intracellular HMGB1 After 48 hours of Exposure
Intracellular HMGB1 is reduced in response to 48 hours of Rottlerin treatment
although it is not detected in the medium. ZR75 cells were untreated (0), treated with
6 μM Rottlerin for 24 or 48 hours, or treated with 500 nM staurosporine (STS) or 1%
H₂O₂ for 16 hours as controls. Immunoblot analyses of HMGB1 in the culture
supernatant (media) and whole cell lysate (WCL) were performed (N=1).
Normalization of HMGB1 to Tubulin was analyzed in WCL only (lower panels).
2.5 Discussion

This section of the dissertation evaluated the mechanism of Rottlerin on cell proliferation of human breast cancer cell lines ZR75 and MCF-7. Rottlerin has been used as a protein kinase Cδ (PKCδ) selective inhibitor on the basis of initial substrate phosphorylation studies in vitro. Several lines of evidence demonstrate that Rottlerin can modulate biological and biochemical events in a PKCδ-independent manner, including those in which it is effective in cells lacking the PKCδ protein. The results of this study show that Rottlerin reduces mitochondrial membrane potential within 1 hour of treatment, followed by subsequent induction of autophagy and ER stress. Ultimately the cells die by a caspase-independent mechanism.

Rottlerin is an antiproliferative agent and it has been reported that the exposure of some cancer cells including glioblastomas and colon cancer cells to Rottlerin promotes apoptosis (Blass et al., 2002; Liao et al., 2005; Lim et al., 2009). In this work we have shown that Rottlerin inhibits breast cancer cell proliferation in a time dependent manner and that this decrease in viability coincides with a decrease in intracellular ATP and loss of mitochondrial membrane potential (MMP). The growth arrest is consistent with Rottlerin acting via uncoupling mitochondria, because classical mitochondrial uncouplers such as FCCP can also promote necrotic cell death. Rottlerin was found to significantly decrease ΔΨm within just one hour of exposure. It is plausible then that the mechanism of Rottlerin-induced cell death begins with an initial insult on the mitochondria, which precipitates a chain of events, including cell death. The decrease in intracellular ATP and
MMP would have significant effects on the cell, affecting all cellular processes that utilize ATP as a cofactor, including the ATP-driven lysosomal proton pump. This would have direct consequences on acidification of the lysosome, and this is evidenced by the inhibition of lysosomal turnover of autophagosomal proteins, LC3-II and p62, in response to Rottlerin in breast cancer cells.

We observed that the pan-caspase inhibitor Z-VAD-fmk blocks caspase activation in breast cancer cells, however, the inhibition of the apoptotic response by Z-VAD-fmk did not prevent Rottlerin-induced cell death. Are we really observing caspase-independent cell death? First, we must define what constitutes caspase-independent cell death, and how cell death can be modulated by caspases. Caspases that participate in apoptosis can be divided into the initiator caspases, which includes caspases 2, 8, 9 and 10, and the effector caspases, caspases 3, 6 and 7. On the basis that pan-caspase inhibitors such as Z-VAD-fmk can reduce the frequency of cells with morphological and biochemical signs of apoptosis (e.g., PARP cleavage and DNA fragmentation), it is inferred that caspase inhibitors exert cytoprotective effects. Therefore, suppression of caspase activity by Z-VAD-fmk results in what appears to be inhibition of apoptosis, if for example PARP cleavage and DNA fragmentation are used as identifying criteria. When other morphological and biochemical criteria of cell death are examined (such as mitochondrial depolarization, phosphatidylserine exposure, or loss of clonogenicity), caspase inhibition frequently does not confer cytoprotection (Kanzawa et al., 2005; Xiang et al., 1996). We conclude then that when breast cancer cells are exposed to a stimulus such as Rottlerin, treatment with the pan-caspase inhibitor Z-VAD-fmk may not protect the cells from cell
death although there is clear evidence that it blocks caspase activation. It is prudent to note that Z-VAD-fmk can inhibit other cysteine proteases such as calpain and cathepsin B, because inhibition of these proteases may induce cell death independent of caspases (Neumar et al., 2003; Waterhouse et al., 1998). Instead of inhibition of caspase activation by Z-VAD-fmk, a potential follow-up experiment would be to overexpress Bcl-2 or knockdown expression of a BH3-only protein in these cells to definitively conclude that Rottlerin does or does not induce apoptotic cell death. Finally, as a caveat to the use of phosphatidylserine (PS) exposure as a marker of early apoptosis, it should be noted that 1) if plasma membranes are permeabilized (as during late apoptosis or early necrosis) Annexin-V can bind to intracellular PS and 2) that PS exposure can be compromised in cells in which autophagy is impaired (Lagasse and Weissman, 1994; Qu et al., 2007).

We also observed upregulation of ER stress proteins BiP and CHOP after treatment with Rottlerin. Protein folding in the ER occurs with the help of several molecular chaperones and folding enzymes. The molecular chaperone BiP/GRP78 (Binding Protein/Glucose Regulated Protein 78) functions by binding to the hydrophobic regions of unfolded or incompletely folded proteins and preventing interactions of these proteins with surrounding molecules (Haas et al., 1992; Munro and Pelham, 1986). BiP/GRP78 possesses ATP hydrolysis activity, which is required to promote protein folding by inducing conformational changes in the unfolded proteins (Hendershot et al., 1996). A cell may experience an increase in the ER client protein load, occurring as a consequence in its physiological conditions. When the ER protein load is excessive in relation to the
ER folding capacity, this may result in the accumulation of misfolded proteins in the ER, causing ER stress. For a cell to overcome ER stress there needs to be coordination between the load of proteins in the ER and the capacity of the ER protein folding machinery. This is achieved by the activation of several signaling pathways, collectively known as the unfolded protein response (UPR). The UPR promotes an adaptive response to ER stress to reestablish homeostasis in the ER. The activation of the UPR in mammalian cells is mediated by three distinct ER stress sensors Inositol-requiring protein-1 (IRE1), activating transcription factor-6 (ATF6) and protein kinase RNA (PKR)-like ER kinase (PERK) (Rasheva and Domingos, 2009). These proteins sense ER stress caused by the accumulation of misfolded proteins and activate downstream signaling effectors in response. While BiP and CHOP were upregulated in response to Rottlerin, activation of JNK was not observed. Stress in the endoplasmic reticulum (ER), induced by perturbations that lead to accumulation of misfolded proteins, also activates JNKs. This contradiction may be resolved by the observation that Rottlerin reduces intracellular ATP, and kinase activity is inhibited. Another possibility is that Rottlerin-induced cell death is caspase-independent. In data not shown, phosphorylation of PERK was also not observed. It is reported that JNK activation releases procaspase-12 from the ER. We know that the cells are not dying by apoptosis therefore release of procaspase-12 is not a probable mechanism of Rottlerin-induced cell death. Taken together, these results indicate that Rottlerin-induced ER stress is most likely mediated by ATP depletion and not the UPR. Accumulation of misfolded proteins in the ER may have toxic effects, ultimately leading to cell death. Because there is no evidence of misfolded proteins in Rottlerin treated cells, we conclude that ER stress is not the major mode of cell death.
The ER stress protein CHOP was found to mediate Rottlerin-induced transcriptional regulation of death receptor 5 (DR5) in human colon carcinoma HT29 cells (Lim et al., 2009). TRAIL induces apoptosis through dimerization of DR4 and DR5, leading to the formation of the FADD complex with the binding of caspase-8 (Jung et al., 2006). However the pan-caspase inhibitor in our experiments should have also blocked caspase-8 activation. This led us to the hypothesis that Rottlerin-induced ER stress may cause necrotic cell death rather than apoptotic cell death.

To test this hypothesis, release of the chromatin protein HMGB1 was examined in Rottlerin treated ZR75 cells. HMGB1 is bound loosely to the chromatin of both interphase and mitotic cells, and is leaked rapidly into the medium when membrane integrity is lost in permeabilized or necrotic cells (Degryse et al., 2001; Muller et al., 2001). We found that HMGB1 is released into the media in H2O2 treated ZR75 cells, whereas none is detected by HMGB1 immunoblotting within 48 hours of Rottlerin exposure in ZR75 cells. However, it is evident that there is a significant reduction in HMGB1 in whole cell lysates treated with Rottlerin. However because HMGB1 cannot be detected in the media, we cannot yet state with certainty that Rottlerin is inducing necrotic cell death. There are few definitive assays available for assessment of necrotic cell death, and this conclusion is typically defined by several morphological and biochemical assays, such as release of heat shock proteins and PARP1 cleavage. Interestingly, PARP1 is also processed during necrosis but a smaller fragment of 50 kD, instead of a major fragment of 85kD, is observed (Gobeil et al., 2001). This event is not inhibited by Z-VAD-fmk, suggesting that proteases are not implicated in the necrotic
cleavage of PARP1. In results not shown, we were not able to detect the 50 kD PARP1 fragment in Rottlerin treated cells. Cell death represents a highly regulated and coordinated process that can follow the activation of distinct but sometimes overlapping biochemical cascades. For example, cells can die as they display apoptotic morphology, (characterized by chromatin condensation and nuclear fragmentation) or a necrotic one (which is associated with a gain in cell volume and cell swelling). Overlapping cell death pathways characterized by both apoptotic and necrotic phenotypes have also been described and such mixed morphologies frequently derive from the activation of separate executioner mechanisms (Elmore, 2007; Galluzzi et al., 2007). Thus, the correct classification of cell death into specific categories in response to the drug of interest may be extremely important for its therapeutic implications. As an example, tumor cells are often resistant to chemotherapeutic regimens that induce apoptosis, but not to necrotic triggers (Guidicelli et al., 2009). In this context, the induction of one specific mode of cell death (i.e., apoptosis), as opposed to another (i.e., necrosis), would result in an obvious therapeutic advantage.

We also found that the autophagic biochemical marker LC3-II was increased within 6 hours in response to Rottlerin in ZR75 and MCF-7 breast cancer cells (Figure 2-22). Rottlerin was reported to increase the presence of autophagic vacuoles in the cytoplasm of pancreatic cells, membrane association of GFP-tagged LC3 to autophagosomes, and a marked induction of LC3-II protein, important hallmarks of autophagy (Akar et al., 2007). Furthermore, inhibition of Beclin-1 expression by RNAi was shown to inhibit Rottlerin-induced autophagy (Akar et al., 2007). Therefore, we hypothesized that Beclin-
1 is required for Rottlerin-induced LC3-II accumulation in breast cancer cells. Contrary to this hypothesis, we found that knockdown of Beclin-1 in ZR75 and MCF-7 breast cancer cells did not have a major effect on LC3-II accumulation. Next, we assessed the induction of autophagy in response to Rottlerin in MCF-7 Control and Beclin-1 knockdown (KD) cells in the absence and presence of lysosomal inhibitors E64d and Pepstatin A. Since the autophagosome is a transient structure, the relative lifetime of LC3-II is short. Therefore, the total levels of LC3-II detected by immunoblot represents the autophagic activity at that specific moment in time. It does not indicate the magnitude of the flux through the autophagic pathway (e.g. autophagosomal delivery of contents to lysosomes). In conditions that induce autophagy, inhibition of lysosomal activity by protease inhibitors such as E64d and Pepstatin A results in enhancement of the amount of LC3-II detected (Asanuma et al., 2003). Therefore simultaneous treatment of Rottlerin plus lysosomal inhibitors would represent the cumulative autophagic activity during the protease inhibitor treatments. The results of this experiment showed that in contrast to stimulation of autophagy in MCF-7 Control cells, Rottlerin did not have a significant effect on MCF-7 Beclin-1 KD cells. This demonstrates that the small positive effect that Rottlerin has on autophagic flux is obstructed in the absence of Beclin-1 expression. These results corroborate evidence that Beclin-1 is required for autophagy induction. Furthermore, transduction of ATG5 shRNA in ZR75 cells also resulted in a significant decrease in the degree of LC3-II accumulation after Rottlerin treatment, as expected, since ATG5 is required for formation of autophagosomes. Furthermore, suppression of ATG5 expression did not have an effect on cell growth or viability compared to non-targeting shRNA control ZR75 cells. The ATG5 KD cells were not
able to survive longer than control cells. Collectively, this suggests that although ATG5 is required for autophagosomal biogenesis, near complete abrogation of autophagy by ATG5 depletion did not rescue cells from Rottlerin induced cell death. Consequently, Rottlerin treated breast cancer cells are not dying by Type II autophagic programmed cell death.

Because of earlier reports that Rottlerin is specific inhibitor of PKCδ (Gschwendt et al., 1994; Keenan et al., 1997; Lu et al., 1997), we performed an experiment to establish whether PKC plays a role in Rottlerin induced autophagy. Wild-type mouse embryonic fibroblasts (WT MEF) and PKCδ-/- MEF were treated with Rottlerin and analyzed by immunoblot for the ability of the cells to induce autophagosome formation. We found that WT MEFs were able to induce autophagosome formation in response to Rottlerin, and the absence of PKCδ expression reduced the ability of Rottlerin to form new autophagosomes by 80%. Therefore we concluded that Rottlerin-induced autophagosome biogenesis is PKC-dependent. However, in addition to the approximate 500 published studies that demonstrate that Rottlerin is a specific inhibitor of PKCδ, there are numerous studies that demonstrate that Rottlerin can modulate biological and biochemical events in a PKCδ-independent manner, including those in which it is effective in cells lacking the PKCδ protein (Soltoff, 2007). While we were able to verify that Rottlerin uncouples mitochondrial respiration, Soltoff also reviews that as a mitochondrial uncoupler, Rottlerin can produce multiple cellular effects that are independent from a direct effect on PKCδ activity including an indirect secondary effect on multiple kinase activities. In addition to blocking PKCδ tyrosine phosphorylation,
Rottlerin blocked the agonist-promoted phosphorylation of multiple proteins, including PKCδ (Ser643), Focal adhesion kinase (FAK, tyrosine), PKCθ (Thr538), protein kinase D (PKD, Ser916), and extracellular-signal-related kinases 1/2 (ERK1/2) (Thr185 and Tyr187). This suggests that Rottlerin is not a specific inhibitor of PKCδ but rather a broad range inhibitor of kinase activity due to a secondary effect of mitochondrial uncoupling. Rottlerin has been used in many studies since it was suggested that it displays specificity as an inhibitor of PKCδ activity in vitro, and on this basis, we were unable to support an absolute conclusion on whether or not Rottlerin is a specific inhibitor of PKCδ in vivo. As an explanation for this discrepancy, we propose several possibilities. PKCδ has been shown to communicate ER stress to the mitochondria and is an essential step in ER stress-mediated apoptosis (Qi and Mochly-Rosen, 2008). Since ER stress is an early response to Rottlerin, the absence of PKCδ might delay the signaling of ER stress-mediated apoptosis in the PKCδ -/- MEF cells. Therefore, rather than Rottlerin directly inhibiting PKCδ activity, the absence of PKCδ activity may simply delay the cell survival response and apoptotic signaling cascade. To determine if this is an accurate postulation, a future experiment treating cells with an ER stress inhibitor such as salubrinol, and immunoblotting for BiP and CHOP upregulation, will clarify whether PKCδ is required for ER stress-induced cell death. Additionally, these confounding effects are sometimes restricted to the mechanism of action of Rottlerin under specific conditions. For example, 10 μM of Rottlerin promoted apoptosis, but 5 μM Rottlerin blocked etoposide-induced apoptosis in glioblastoma cells (Blass et al., 2002; Jane et al., 2006). Finally, MEF cells, like their name suggests, are normal immortalized fibroblasts that secrete and assemble their own extracellular matrix. In data not shown, autophagic
flux in also inhibited in response to Rottlerin in U251 glioblastoma cells. For that reason, a contributing factor to the disparity in these results may be attributable to cell type differences between normal and malignant cell lines. To prove this, a study would need to be performed by generating ZR75 PKCδ KD cells.

A summary of the timeline of cellular events observed in Rottlerin-treated breast cancer cells is detailed in Figure 2-23. Rottlerin reduces mitochondrial membrane potential and decreases intracellular ATP within 1-hr of exposure to breast cancer cells. This in turn causes upregulation of ER stress proteins and stimulates autophagy, a cell survival response. The decrease in ATP causes an inhibition of lysosomal degradation; meanwhile ER stress induces hallmarks of apoptosis, including PARP cleavage. However the cells die from a caspase-independent mechanism, shown by the inability of Z-VAD-fmk to prevent Rottlerin-induced cell death. Therefore the cells are not dying by Type I apoptotic PCD. Cells are not undergoing Type II autophagic PCD either, as evidenced by inhibition of the autophagy pathway by KD of ATG5. Therefore cells are most likely undergoing Type-III necrotic cell death, a probable end result from the early depletion of ATP.
Figure 2-23: Summary Schematic of the Effect of Rottlerin on Breast Carcinoma Cells. Rottlerin reduces mitochondrial membrane potential and decreases intracellular ATP. This in turn causes upregulation of ER stress proteins and stimulates autophagy, a cell survival response. The decrease in ATP causes an inhibition of lysosomal degradation, and ER stress induces hallmarks of apoptosis, however this causes cells to die from a caspase-independent mechanism. Therefore cells are most likely undergoing necrosis as a probable result from the depletion of ATP.
2.6 Conclusions

1. Rottlerin is an antiproliferative and cytotoxic agent in ZR75 and MCF-7 breast cancer cells.

2. Rottlerin reduces the ability of these breast cancer cells to form colonies in soft agar, a measure of anchorage-independent growth.

3. Rottlerin induces the apoptotic pathway within 48 hours of exposure, as evidenced by caspase activation and Annexin-V staining of the phospholipid bilayer.

4. The pan-caspase inhibitor Z-VAD-fmk blocks caspase activation in breast cancer cells, however, the inhibition of the apoptotic pathway by Z-VAD-fmk did not rescue the cells from Rottlerin-induced cell death.

5. The autophagic biochemical marker LC3-II was increased within 6 hours in response to Rottlerin in ZR75 and MCF-7 breast cancer cells.

6. Rottlerin caused a deregulation of the autophagy pathway as evidenced by an inhibition of the lysosomal turnover of LC3-II and p62/SQSTM1. This occurs together with a modest increase in autophagosome biogenesis.

7. The increase in autophagosome formation that is occurring in response to Rottlerin is inhibited by suppression of Beclin-1 expression by RNAi, indicating that Beclin-1 is required for autophagosome biogenesis.

8. ATG5 causes a decrease in LC3-I conversion to LC3-II in Rottlerin treated breast cancer cells, indicating that ATG5 is required for autophagosomal biogenesis.
9. Knockout of PKCδ in mouse embryonic fibroblasts attenuated the autophagic response to Rottlerin.

10. Rottlerin reduces mitochondrial membrane potential (MMP) within 1 hour of exposure, indicating that an early reduction in ATP levels may trigger the changes in the autophagy pathway.

11. This early insult of Rottlerin exposure on mitochondrial respiration is followed by induction of ER stress, measured by increases in CHOP and BiP/Grp78.

12. Although HMGB1 cannot be detected in media of ZR75 treated cells, Rottlerin reduces intracellular HMGB1 in these lysates. HMGB1 release is an indicator of necrotic cell death; therefore, more studies are required to conclude whether or not Rottlerin induces necrotic cell death.

13. In conclusion, Rottlerin induces caspase-independent cell death by reducing MMP, stimulating ER stress, and inhibiting autophagy, a cell survival response.
2.7 Summary

Rottlerin has been shown to be an antiproliferative agent in glioblastoma and colon cancer cells by promoting apoptosis (Blass et al., 2002; Liao et al., 2005; Lim et al., 2009). In this work we have shown that Rottlerin inhibits breast cancer cell proliferation in a time dependent manner and that this decrease in cell growth coincides with the apoptotic response. The apoptotic response was detected after ER stress and deregulation of the autophagy pathway. We also observed that the pan-caspase inhibitor Z-VAD-fmk blocks caspase activation in breast cancer cells, however, the inhibition of caspase activation by Z-VAD-fmk did not rescue the cells from Rottlerin-induced cell death. This led us to the hypothesis that Rottlerin-induced ER stress may cause necrotic cell death rather than apoptotic cell death. To test this hypothesis, release of the chromatin protein HMGB1 was examined in Rottlerin treated ZR75 cells. We found that HMGB1 is released into the media in H$_2$O$_2$ treated ZR75 cells, whereas none is detected by HMGB1 immunoblotting within 48 hours of Rottlerin exposure in ZR75 cells. However, it is evident that there is a significant reduction in HMGB1 in whole cell lysates treated with Rottlerin.

Rottlerin was reported to increase the presence of autophagic vacuoles in the cytoplasm of pancreatic cells, membrane association of GFP-tagged LC3 to autophagosomes, and a marked induction of LC3-II protein, important hallmarks of autophagy (Akar et al., 2007). However we found that most of the accumulation of LC3-II that occurs with Rottlerin treatment is due to a block in autophagosome turnover at the lysosomal fusion
step, and not to increased autophagosome biogenesis. Furthermore, inhibition of Beclin-1 expression by RNAi was shown to inhibit Rottlerin-induced autophagy (Akar et al., 2007). Therefore, we hypothesized that Beclin-1 is required for Rottlerin-induced LC3-II accumulation in breast cancer cells. Contrary to this hypothesis, we found that knockdown of Beclin-1 in ZR75 and MCF-7 breast cancer cells had little effect on LC3-II accumulation. Next, we assessed the induction of autophagy in response to Rottlerin in MCF-7 Control and Beclin-1 knockdown (KD) cells in the absence and presence of lysosomal inhibitors E64d and Pepstatin A. The results of this experiment showed that in contrast to the modest stimulation of autophagy in MCF-7 Control cells, Rottlerin did not have a significant effect on autophagosome biogenesis in MCF-7 Beclin-1 KD cells. This demonstrates that the small positive effect that Rottlerin has on autophagic flux is obstructed in the absence of Beclin-1 expression. These results corroborate evidence that Beclin-1 is required for autophagy induction. Furthermore, transduction of ATG5 shRNA in ZR75 cells also resulted in a significant decrease in the degree of LC3-II processing after Rottlerin treatment, and was evident even before co-treatment with lysosomal inhibitors. Consequently, Rottlerin treated breast cancer cells are not dying by autophagic cell death.

Rottlerin has been used as a protein kinase Cδ (PKCδ) selective inhibitor on the basis of initial substrate phosphorylation studies in vitro. Several lines of evidence demonstrate that Rottlerin can modulate biological and biochemical events in a PKCδ-independent manner. Because of earlier reported literature that Rottlerin is specific inhibitor of PKCδ (Gschwendt et al., 1994; Keenan et al., 1997; Lu et al., 1997), we performed an
experiment to establish whether PKC plays a role in Rottlerin induced autophagy. Wild-type mouse embryonic fibroblasts (WT MEF) and PKCδ -/- MEF were treated with Rottlerin and analyzed by immunoblot for autophagic flux. We found that WT MEFs were able to induce autophagic flux in response to Rottlerin, but PKCδ -/- MEF displayed a blunted response in Rottlerin-induced autophagosome biogenesis. These results suggest that Rottlerin-induced autophagy is in part PKC-dependent. However, in addition to the approximate 500 studies including ours that demonstrate that Rottlerin is a specific inhibitor of PKCδ, there are numerous studies that demonstrate that Rottlerin can modulate biological and biochemical events in a PKCδ-independent manner, including those in which it is effective in cells lacking the PKCδ protein.

In summary, the results of this study show that Rottlerin reduces mitochondrial membrane potential within 1 hour of treatment, followed by simultaneous inhibition of autophagosome fusion with lysosomes, and induction of ER stress, which ultimately leads to caspase-independent cell death. The modest induction of autophagosome biogenesis that occurs with Rottlerin treatment is likely to represent an attempt on the part of the cells to compensate for the initial decline in ATP production. However, because autophagosomes fail to fuse with lysosomes, autophagy is unable to overcome the bioenergetic deficiency caused by the initial mitochondrial insult of Rottlerin.
References


inhibit mammalian target of rapamycin (mTOR)-mediated downstream signaling.


